University of Alberta

Cyclosporine immunosuppression: The role of calcineurin phosphatase inhibition

by

Thomas David Batiuk ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Cyclosporine immunosuppression: The role of calcineurin phosphatase inhibition by Thomas David Batiuk in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences - Medicine.

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To Karen, Andrew and Elizabeth,

Physician, scientist, traveler - these words describe some of the activities that I have enjoyed at different times, and with a variety of different people.

"Husband" and "father" are different. They are titles given to me by you. They define who I am and who I will always want to be.

Abstract

Cyclosporine (CsA) is a potent immunosuppressant but its mechanism of action in patients is unknown. CsA *in vitro* prevents the activation of T lymphocytes by inhibiting the serine-threonine phosphatase activity of the calcium-regulated enzyme calcineurin (CN). Our hypothesis was that CsA is immunosuppressive in patients because it inhibits CN activity in T lymphocytes.

We found that CN activity in peripheral blood leukocytes (PBL) from CsA-treated adult and pediatric renal transplant and adult cardiac transplant patients was reduced compared to values in healthy controls. Pharmacodynamic monitoring of 68 adult renal transplant recipients showed that at trough CsA blood levels of 183 ± 109 μg/L, CN inhibition was 51 ± 32% and peaked at 70 ± 20% concomitant with peak CsA levels (587 ± 401 μg/L). In contrast, CsA *in vitro* completely inhibited CN activity in control PBL at 100 μg/L, with an *in vitro* IC_{50} of 10 μg/L. This *in vitro/in vivo* discrepancy was not due to recovery of samples during sample preparation. Indeed, in vitro experiments showed that CsA efflux from PBL, required for CN recovery, was very slow in typical culture conditions. Finally, while thought to act primarily on T lymphocytes, CsA inhibition of CN activity occurred equally in all the blood cell types present in PBL preparations.

We then determined the relationship between CN inhibition and immune responses in mice rejecting an intraperitoneal allogeneic tumor (P815 mastocytoma cells). Each dose of orally administered CsA caused a similar

degree of inhibition of both CN activity and IFN-γ induction in spleen preparations. To test the molecular basis for this relationship, we tested the effect of partial CN inhibition on the molecular events that immediately follow CN activation. We found that the degree to which CsA inhibited CN activity led to a similar degree of inhibition of NFATp dephosphorylation, NFAT-like DNA binding and promoter activation, IFN-γ and IL-2 mRNA induction, IFN-γ secretion and PBL proliferation during a mixed lymphocyte reaction. These data suggested that CN activity was rate limiting for the activation of some T lymphocyte cytokine genes and for antigen-stimulated proliferation.

We propose that the immunosuppressive mechanism of action of CsA in transplant patients is the partial inhibition of CN activity, a rate limiting enzyme for lymphocyte response to antigenic stimulation. This model would account for both the observed reduction in acute rejection rates as well as the ability of CsA-treated patients to respond to antigenic challenges.

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List of Abbreviations

Ab	antibody
Ag	antigen
APC	antigen presenting cell
CAT	chloramphenicol acetyltransferase
CDR	complementarity determining regions
CN	calcineurin
CsA	cyclosporine
CyP	cyclophilin
DAG	diacylglycerol
DTH	delayed type hypersensitivity
ELISA	enzyme linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
FKBP	FK-506 binding protein
GVHD	graft versus host disease
HPLC	high performance liquid chromatography
IC_{50}	concentration at which 50% of activity is inhibited
Ig	immunoglobulin
IP_3	inositol 1, 4, 5 trisphosphate
MLR	mixed lymphocyte reaction
Neo	Neoral® formulation of oral cyclosporine

NFAT	nuclear factor of activated T cells
NK	natural killer
PBMC	peripheral blood mononuclear cells
PGP	P glycoprotein
PKA	protein kinase A
PLC	phospholipase C
RABA	radioactive antibody binding assay
RBC	red blood cell
RHD	Rel homology domain
RIA	radioactive immunoassay
RT-PCR	reverse transcriptase polymerase chain reaction
SIM	SandImmune® formulation of oral cyclosporine
TCR	T cell receptor

Chapter One

Background

I. Transplantation - Acceptance And Rejection

Transplantation is the act of transferring cells, tissues or organs from one site to another. Allotransplantation is the transfer of tissue between genetically different members of the same species and is the treatment of choice for dozens of diseases involving the kidney, heart, liver, lung, cornea, pancreas, bone marrow and small intestine. While challenges remain, the transplantation of a variety of organs from a living or recently deceased person to another, often critically ill person is now considered a relatively straightforward procedure.

However, despite its stature as a routine treatment option, transplantation is still a young science and relatively recent practice. The first clinically successful procedure was a kidney transplant that took place between monozygotic twins in 1954 (1). A kidney was the obvious choice for a transplanted organ since most people have two, and evidence existed that individuals could live with only one, whether due to congenital or surgical absence of a second. Identical twins were chosen because it had been demonstrated that skin transplants between genetically identical individuals survived indefinitely (2), raising the possibility that other organs might similarly be "accepted" by the recipient. Indeed, in this first case report, preparations for kidney transplantation included experimental transplantation of skin from the donor twin. The success of the skin transplant emboldened the clinicians and patients involved to proceed with the kidney transplant, the success of which was, even by current standards, virtually complete. The kidney functioned immediately and the biochemical and clinical abnormalities caused by renal failure normalized quickly. Because of persistent hypertension, the transplant recipient required nephrectomy of both diseased organs and continued to be clinically stable, further proving the function of the transplant.

Owing to the scarcity of identical twins, the unqualified success of this transplant was difficult to duplicate until the development and use of immunosuppressive drugs. In 1963, both glucocorticosteroids and azathioprine (a thiopurine derivative) were reported to successfully prevent or reverse allograft rejection (3,4). The latter series also included the first cadaveric renal allograft to successfully function for longer than one year. These studies produced an optimism that the technical hurdles of transplantation from cadavers could be overcome and the immunologic difficulties of rejection could be controlled. This led to attempts at transplanting livers (5) and hearts (6). However, despite the enthusiasm of a group of surgical mavericks (now referred to as transplantation pioneers) and the promise of chemical immunosuppression, widespread success in transplantation came slowly and only with a greater understanding of the mechanisms involved in allograft rejection.

Key observations in the 1940s by P.B. Medawar suggested that allograft rejection was an immunologic phenomenon. Working with burn victims, he observed that autografts (from one site to another on the same patient's body) uniformly survived, whereas allografts from relatives often failed. Repeat transplantation from the same donor following a first rejection resulted in a more rapid and vigorous rejection response than the first (7). In subsequent animal experiments, he showed that this "second set" rejection occurred only with skin from the same donor, but not with genetically different animals (8). These results identified two hallmarks of the adaptive immune response: specificity and memory. In further studies, Billingham, Brent and Medawar showed that, in contrast to this "actively acquired immunity," mice and chickens could learn to not reject foreign skin grafts if they had been exposed to cells from the donor during fetal life (9). They dubbed this phenomenon "actively acquired tolerance" and indicated that education of the immune system could result in an enhanced response upon restimulation, or antigen-specific tolerance. Some of the cells and molecules involved in an immune response are discussed below.

II. Of Cells And Molecules

The cellular infiltrate of a rejecting organ contains both antigen-specific lymphocytes as well as non-specific cells, such as macrophages. Macrophages make numerous proinflammatory factors, such as cytokines, growth factors, eicosanoids, enzymes and procoagulant activities that are likely to contribute to the cell injury and dysfunction in vascularized grafts. Additional non-specific effector mechanisms include the coating of foreign proteins and viruses by proteins of the complement and collectin systems, which can lead to inactivation and/or phagocytosis. Most early cellular graft injury is probably due to activation of lymphocytes bearing specialised antigen specific cells, the T-cell receptor and immunoglobulin on the membranes of T and B lymphocytes, respectively, and the interactions of these cells with specialized antigen presenting cells expressing the major histocompatibility complex molecules. Some of the details of these cells and receptors will be described.

B lymphocytes and Immunoglobulin

B lymphocytes are bone marrow derived mononuclear cells that recognize antigens by virtue of the expression of immunoglobulin (Ig). Each Ig is composed of two membrane anchored 50 kDa "heavy" chains, connected by two disulfide bonds, each of which is attached to one of the two 25 kDa "light" chains (10). An individual B lymphocyte will express many copies of an identical, clonally derived Ig. Each Ig has a rather narrow antigen specificity but because each Ig is unique, a population of B cells will provide an individual with the ability to recognize an apparently limitless array of foreign antigens. This polymorphism is largely due to the coding of Ig genes by multiple gene segments, each with multiple distinct coding regions. Transcription occurs only after they undergo rearrangement into functional genes. The fact that only one codon from the many available within each gene segment is expressed makes it possible to generate 10^7 to 10^8 functional gene rearrangements. In addition to genetic rearrangement, somatic mutations occur to increase the variability of the final gene products. Virtually all of the variability is contained within the "complementarity determining regions" (CDRs) which form the antigen contact sites. The six CDRs - three each from the light and heavy chains - determine the antigenic specificity (10,11).

When Igs on the surface of a B lymphocyte are cross-linked by binding to antigen, this partially activates the cell. If, within the next 24-48 hours, the B lymphocyte receives other activation signals by activated helper T lymphocytes, the cell will enter the cell cycle, proliferate and eventually differentiate into an effector plasma cell. Plasma cells generate huge quantities of secreted Ig that bind to antigen, inactivating it or targeting it for phagocytosis. The requirement for T lymphocyte help acts as a safety measure to control B lymphocyte proliferation and differentiation.

Antigen presenting cells and the MHC

In contrast to B lymphocytes, T lymphocytes do not recognise antigen in its native conformation but rather as a peptide fragment expressed in the context of self-MHC on antigen presenting cells (APCs). These genes encode the strong transplantation antigens, the class I and II MHC molecules that are responsible for binding intracellular peptides and displaying them for recognition by T lymphocytes. The MHC alleles are highly polymorphic (12) and are codominantly expressed. These factors account for the diversity of antigens that can be recognised within a given population. Class I is expressed on most cells but class II has a more restricted tissue distribution, being confined to specialised APCs.

Class I and II molecules are both heterodimers and share, based on their crystal structures, some general structural similarities (13-15). Both have short intracellular tails with no identified functions, transmembrane anchors and two nonpolymorphic membrane proximal Ig-like domains and, finally, two membrane distal "sheet and helix" domains. The 4 stranded β-pleated sheet from each domain abuts the other forming an 8 stranded platform, surrounded on two sides by the parallel α helices, one contributed from each domain. This structure forms a groove, which is occupied by a linear polypeptide antigen. The polymorphisms seen in the MHC genes affect the bases that encode amino acids in the peptide binding groove and likely occurred by interallelic segmental exchange (16-18). These residues interact with the amino acid side chains of peptides, and will thus influence the ability of different residues, and hence polypeptides, to bind stably within the groove.

The class I groove accommodates short peptides of about nine amino acids, and the class II groove accommodates longer peptides - 13-25 amino acids. In addition to size differences, the peptides are also derived from different intracellular compartments. Class I molecules bind peptides from endogenous proteins, presumably to sample for (intracellular) viral infection, during assembly in the ER (19,20). Class II molecules present peptides primarily from exogenous proteins (19).

In addition to MHC, APCs express other non-polymorphic surface proteins that act to stabilise the interactions with T lymphocytes and, in some cases, activate independent signal transduction pathways. These “second signals” are necessary for full T lymphocyte activation and include certain adhesion molecules of the Ig superfamily. For example B7-1 and B7-2 on the APC engage CD28 on the T cells activating systems in the T cell which synergize with the signals from the T cell receptor (21-23). CD28 ligation activates proteins that regulate both IL-2 transcription and IL-2 mRNA half life (24). In CD28 knockout mice, T cell triggering can still occur, indicating that other systems can compensate (25). Other interactions with the APC which could contribute to signalling include other adhesion molecule ligand receptor pairs, such as ICAM-1/LFA-1 and LFA-3/CD2, and cytokines such as IL-1 and IL-6, produced by the APC. Thus stimulation of the primary T cell response appears to be initiated by high density of the allogeneic class II molecules on the APCs and supported by secondary signals from cytokines and adhesion molecules. The signals from the triggered CD4 T cell then activate the APC to increase the signals to the T cell in a cascade of reciprocal activation.

T lymphocytes and the T cell receptor

As noted above, MHC/peptide complexes on APCs are recognized by T cell receptors (TCRs) on T lymphocytes. The TCR is a dimer of non-identical α and ß chains, or less commonly γ and δ chains. Each TCR chain resembles an Ig light chain, having V and C regions, with the addition of a membrane anchor and intracytoplasmic region. Like the Ig V region, (26), each V region contains three hypervariable CDRs which are thought to engage the MHC-peptide complex. The C region is more proximal to the membrane and is likely structural in nature. The V regions of the α and β chains dimerize to form the ligand binding region. It is likely that all six CDRs of the TCR engage the upper surface of the MHC (27,28). The outer regions of the TCR (CDRs 1 and 2) engage the α-helices of the MHC and the central region (the CDR3s) engage the peptide (29,30). The affinity of the TCR:MHC-peptide binding reaction is quite low (31). This low affinity may permit rapid "on" and "off" rates, permitting a single MHC-peptide complex to serially trigger many TCRs (32).

In addition to TCRs, mature T cells express either one of the coreceptors CD4 or CD8. Both molecules are transmembrane proteins with no identified intrinsic enzyme activity. The presence of CD4 molecules indicates that the TCR for that cell will interact only with MHC class II molecules, while CD8+ T lymphocytes bear TCRs that interact only with MHC class I molecules. These distinctions are broadly helpful in determining whether activated T lymphocytes will behave as "helper" T cells (expressing CD4) or "cytotoxic" T cells (CD8+), although there is overlap in the phenotypes of these cells.

Unlike TCRs and MHC molecules, the coreceptors CD4 and CD8 are nonpolymorphic and invariant. They each bind a nonpolymorphic region of their respective MHC ligands (33,34). It has been suggested that a primary role for CD4 or CD8 is to increase TCR interaction with MHC by stabilizing binding (35). However, the findings that non-receptor kinases are linked to the cytoplasmic tails of CD4 and CD8 (36), and the demonstration that CD4 associates with the

TCR only under conditions of activation suggest a more fundamental role in signaling for these coreceptor molecules (37).

How allorecognition occurs *in vivo* is not clear. Small numbers of amino acid differences in the donor MHC can lead to strong responses. The binding of sufficient TCRs to MHC molecules is a necessary condition for T cell activation by antigen. By an unidentified mechanism, the signal is transduced by CD3, a multi-transmembrane protein complex (38). An early event following TCR stimulation is an increase in tyrosine kinase activity (39). CD4, CD8 and the CD3 complex are all associated with tyrosine kinases, including ZAP, $p56^{lck}$ and $p59^{fyn}$. These kinases phosphorylate the tyrosine residues in the CD3 complex, in key transduction molecules, and in one another, resulting in the activation of many signalling pathways leading, within minutes, to gene transcription.

A particularly important signalling pathway involves the activation of phospholipase C (PLC)-γ_1, which lyses the membrane phospholipid phosphatidyl inositol bisphosphate to yield the second messengers inositol 1, 4, 5 trisphosphate (IP_3) and diacylglycerol (DAG) (reviewed in (40)). DAG activates protein kinase C (PKC) leading to the activation of the transcription factor NF-κB (Figure 2). Binding sites for NF-κB are found in the promoters of several genes activated following T lymphocyte stimulation. However, a recent report of a mouse lacking a lymphocyte specific form of PKC did not report any impact on T lymphocyte signalling (41). Instead, the mice were observed to manifest some B lymphocyte functional abnormalities.

The other product of phosphatidyl inositol bisphosphate hydrolysis is IP_3 which binds to receptors on the ER to release stored calcium and raise cytosolic Ca^{2+} levels. These levels are sustained by increased calcium entry through channels in the plasma membrane to maintain high cytosolic Ca^{2+} concentrations (42,43). High intracellular calcium activates calcium-regulated enzymes, including the enzyme calcineurin (CN). CN dephosphorylates a cytosolic factor called NF-ATp, which is free to translocate from the cytosol to the nucleus (44,45). When cytoplasmic and nuclear factors assemble to form the full NF-AT complex, transcription of IL-2 mRNA begins. While the five NF-AT sites account for the majority of inducible IL-2 expression, it has been thought that the NF-κB site is also critical (46). The characteristic behaviour of the IL-2 gene requires the interaction of multiple transcription factors binding to these sites.

A final intracellular signal transduction pathway believed to be important in lymphocyte gene activation is the Ras-MAP kinase pathway (47,48). Stimulation of the TCR by antigen can lead to the activation of a cascade of protein-protein interactions, between molecules such as Shc, GRB2, Sos (40), that result in Ras activation. Ras has been shown to be necessary for TCR signal transduction *in vitro* (49). Ras activation can lead to the activation of the ERK members of the MAP kinase family of protein tyrosine kinases (50). ERK activation results in the activation of the Jun N-terminal kinases (JNKs) (51), which then activate the transcription factor c-Jun. Finally, the Ras-MAP kinase pathway may play a role in the activation of the Ets family of transcription factors (52). Despite the wealth of literature, the precise *in vivo* physiologic role of this signalling pathway or any of the individual proteins is not established.

Naive CD4 T cells make predominantly IL-2 in their first encounter with antigen, whereas previously stimulated or memory T cells make other cytokines. IL-2 engages its receptor, committing the cell to activation, differentiation, mitosis, and eventually clonal expansion. Effector functions, such as cytotoxicity in CD8 cells, emerge followed by the appearance of molecules associated with memory and recirculation (53).

Mechanisms of graft rejection

Understanding these molecules and cells has helped make sense of the clinical and histopathologic characteristics of transplant rejection. Transplant rejection is generally classified into three types - accelerated/hyperacute, acute and chronic. Each type has a characteristic clinical and histopathologic pattern, and the immune mechanisms mediating each type have been, to a greater (hyperacute) or lesser (chronic) extent, characterized.

I. Accelerated and Hyperacute Rejection

Hyperacute rejection is mediated by preformed antibodies against HLA class I molecules, ABO blood group antigens (54), or less commonly, poorly defined endothelium specific antigens of arteries (55). The antibodies on the endothelium fix complement, which attracts polymorphs, and destroy the endothelium within minutes to hours. Alternatively, a recipient previously sensitised to donor Ag by pregnancies, transfusions, or previous grafts may not have preformed antibodies in the circulation. However, the rapid return of high levels of specific T cells and/or alloantibody directed against the Ag of the graft can result in a vigorous rejection after 48-72 hours, earlier than typical acute rejection.

II. Acute Rejection

Around 5-7 days after transplantation, the tissue begins to manifest signs of two processes: inflammation and specific cell injury and death. The inflammation is manifest by infiltration with polymorphonuclear leukocytes, accompanied by edema and reduced blood flow. Specific destruction of parenchymal and endothelial cells by infiltrating lymphocytes, coupled with decreased perfusion, causes a rapid loss of function. Destruction of blood vessels frequently leads to late infarction of some or all of the tissue.

The inflammation in the graft is analogous in some respects to the delayed type hypersensitivity reaction (DTH), exemplified by the classic skin reaction to tuberculin. DTH is an *in vivo* phenomenon with no single *in vitro* correlate. It is manifest histologically as a heterogeneous non-specific inflammation, with edema, fibrin accumulation, T cell infiltration (both specific and non-specific), B cells, numerous macrophages, and lesser numbers of other leukocytes, and endothelial changes. The key events in DTH are cytokine production (especially TNF-α and ß, IFN-γ, and IL-1), altered expression and function of adhesion molecules, and the activation of many other cells, particularly macrophages. Although usually ascribed to $CD4^+$ T cells, DTH reactions mediated by CD8 T cells have been described. The result is graft inflammation.

T lymphocytes are critical for cell mediated rejection. This is based on the observations that nude mice, which lack T lymphocytes, will not reject a transplanted organ, even those from other species. Also, in bone marrow transplantation studies, removing T lymphocytes from the donor marrow prior to transplantation eliminates graft versus host disease. The first entry of T lymphocytes into the allograft probably occurs by a combination of non-specific and specific interactions with endothelial cells. Antigen non-specific cells may be attracted by endothelium activated by non-immune injury from the transplant donor, the surgery, or the preservation. Antigen specific T cells probably interact with donor APC in the organ or in the host for their primary stimulation, and begin the process of activating the endothelium. Sensitised lymphocytes can then interact with the allogeneic endothelium both to infiltrate the tissue and eventually to damage the endothelium.

The candidate mechanisms of specific cell injury include Fas-induced apoptosis, cytotoxic alloantibody and receptor-directed exocytosis of cytokines, serine esterases and perforins. Fas expression is upregulated in animal models of graft versus host disease (GVHD) (56), and the absence of Fas is associated with lymphoproliferative disorders (57,58). Serine esterases are expressed in the infiltrate of rejecting grafts (59), although perforin knockout mice show only partial impairment of cytolytic activity and no impairment of graft rejection (60). Numerous cytokines are found in the infiltrate of rejecting grafts or in the serum, but the roles of these mediators are not established (61). Some may cause injury, but some may reflect a response to injured tissue. Both CD4 and CD8 T cells are present in rejection and neither has an exclusive role (62).

III. Chronic Rejection

An initially successful transplant may gradually lose its function in a slow scarring process. The arteries become obstructed by intimal thickening, and the graft undergoes progressive parenchymal atrophy and interstitial fibrosis. Chronic rejection, best studied in renal and heart transplant recipients, can occur months to years after transplantation. In some cases it may be antibody-mediated, but typically no antibody is demonstrable and the pathogenesis is not understood (63).

Thus the immune system has many defence mechanisms against foreign tissue. Hyperacute rejection is usually prevented by "crossmatching" the recipient's serum for complement-fixing antibodies against donor lymphocytes (54). In contrast, inhibition of cellular rejection continues to be the primary immunologic challenge in clinical transplantation. Matching MHC antigens to minimise the chance of immune recognition is widely practised in renal transplantation, to the point of being legislated in the U.S. However, owing to the codominant expression of multiple MHC alleles by every individual, the contribution of tissue typing to clinical transplantation has been trivial (64).

Successful immunosuppressive strategies usually suppress primary alloantibody as well as T cell responses, probably by suspending help from CD4 T cells, but do little to preformed antibody and may have difficulty suppressing secondary antibody responses. Thus, inhibition of an immune response, in an Ag-specific manner has been, and continues to be the holy grail of immunosuppression.

In a review of his discovery of cyclosporine (CsA), Borel identified three phases in the development of therapeutic immunosuppression (65). In his first phase, or generation, he identified antiproliferative agents such as cyclophosphamide, azathioprine and methotrexate, that worked by preventing cell division. Unfortunately, these agents were indiscriminant cytotoxic against all dividing cells, including immunocompetent cells. The second phase included the use of total lymphoid irradiation and drugs like L-asparaginase, glucocorticosteroids as well as antilymphocyte serum raised in animals such as rabbits and horses. Although these treatments were considered more specific for lymphoid cells, their effects were still indiscriminant and chronic administration, as evidenced with steroids, was associated with significant non-lymphoid toxicity. Borel considers the third phase to be the development of agents that modulate specific subpopulations of immunocompetent cells, starting with the discovery of the fungal metabolite cyclosporine (65).

III. Cyclosporine

In 1969/70, the Sandoz pharmaceutical company identified two new strains of fungi. Extracts from these fungi showed *in vitro* antifungal properties. For technical reasons, the strain *Tolypocladium inflatum* Gams was used for the preparation of a mixture, designated 24-556, for initial biologic screening. The active compound was shown to be a small neutral peptide and was named cyclosporine (CsA). Borel showed that CsA could inhibit a variety of humoral and cellular immune responses, including the prevention of skin graft rejection and graft versus host disease in mice (66,67). However Borel later noted that an important reason for developing CsA clinically was its limited toxicity, particularly its weak myelotoxicity (65). This latter point indicated that CsA was not inhibiting cell division. Thus, its immunosuppressive mechanism was due to a mechanism not previously recognized.

Because of its potent immunosuppressive activity and comparatively trivial toxicity profile in animal models, CsA moved quickly into clinical trials with clinical trials beginning in two centers in June 1976. Both studies, one in cadaveric renal allograft recipients (68) and the other in bone marrow recipients (69) showed that CsA exerted strong immunosuppressive activity. Large multicenter clinical trials in Europe and Canada showed significant improvement in renal graft outcome (70,71) that persisted for 3-5 years (72). Liver graft survival in adult recipients went from 30-35% at one year to 58-70% after five years (73). Similarly, cardiac transplant recipients treated with CsA enjoyed a

20% increase in one year survival (74). In addition, rejection episodes were less severe and less frequent in CsA-treated patients, contributing to a 40% reduction in both the number of days a patient was in hospital as well as the cost of cardiac transplantation.

The impact of these results on clinical care was revolutionary. While kidney transplantation had achieved the status of being an acceptable treatment option, the transplantation of other organs was still considered largely experimental. The reduction in graft rejection, and improvement in short survival, led to a fundamental change in the clinical management of many diseases.

In the first report on the efficacy of CsA in clinical renal transplantation, three side effects were reported: hirsutism, hepatotoxicity and nephrotoxicity (68). With experience, other dose dependent toxicities were identified, including hypertension, glucose intolerance and gingival hyperplasia. In addition, a variety of apparently idiosyncratic syndromes, affecting primarily the vascular, hematologic and central nervous systems, have been described (71,75). Hypertension is a clinically important toxicity because of its high frequency and its association with the development of ischemic heart disease and stroke. Nephrotoxicity is also common, and presents clinical challenges in kidney transplant recipients inasmuch as acute nephrotoxicity can be difficult to distinguish from acute rejection and chronic CsA nephrotoxicity shares many histopathologic features with chronic rejection.

Chemistry and pharmacology

CsA is a neutral, hydrophobic, cyclic 11 amino acid peptide of 1203 molecular weight (Figure 1). As shown, 7 amino acids are N-methylated (positions 1,3,4,6,9,10,11) including a novel 9 carbon unsaturated amino acid found at position 1. CsA is a rigid and stable structure because of the formation of intramolecular hydrogen bonds between amino acids 2 and 5 (two bonds), 6 and 8, and 7 and 11. Because of the isolation of other, related fungal metabolites, the original cyclosporine was renamed cyclosporine A.

The side effects seen in CsA-treated patients introduced the need for some sort of monitoring. Radioimmunoassay (RIA) and high performance liquid chromatographic (HPLC) technics permitted the widespread monitoring of circulating CsA levels (76,77) and allowed the study of the kinetic distribution and metabolism of CsA. The measurement of CsA blood levels remains the only widely accepted method of measuring CsA effect, as all other attempts at determining a predictive bioassay have failed.

The measurement of CsA blood levels has been valuable in indicating the huge variability in the dose-concentration relationship for CsA between individuals, as well for measurements in the same person. Sources of this pharmacokinetic variability include variability in absorption, distribution and elimination. The absorption of the conventional oral CsA formulation is poor, varying between 10% and 70% and is bile-dependent, so that conditions that change bile flow (such as liver disease or the nature of the carrier solution for

CsA) can markedly change absorption (78,79). Distribution within circulating blood is affected by the volume of erythrocytes and the protein and lipid concentrations (80-82), each of which can vary considerably over time. Finally, elimination of CsA occurs primarily by metabolism in the liver (83,84) and can be altered by dozens of drugs (85). Each of these factors may change over the clinical course of a patient and accounts for the absence of an accepted, standardized method for determining, in an individual patient, whether they are receiving too much, too little, or the right amount of CsA.

How does it work?

Determining the "right amount" of CsA to administer implies knowing, at a minimum, its effect on a target population of cells. Although a large body of literature addressing this issue rapidly accumulated, many early studies yielded conflicting, if not outright contradictory results (reviewed in (75,86,87)) owing, at least in part, to an incomplete knowledge of the clinical pharmacokinetics of CsA. Indeed, some early *in vitro* studies used CsA concentrations 100X higher than those ever achieved in patients. Even Borel's first *in vivo* mouse data used oral CsA concentrations 30X higher than those first used in patients (66).

By the mid 1980s accumulated evidence suggested that CsA inhibited the activation of naive helper T lymphocytes. The support for this hypothesis included the lack of stem cell toxicity, the lack of effect on memory immune responses (88,89) and the inhibition of the generation, but not the effector function, of cytotoxic T lymphocytes (90,91). Furthermore, the observations that CsA inhibited the transcription of growth and differentiation factors (such as IL-2 and IFN-γ) by T lymphoblasts (92-95) were supported by clinical data showing a reduction in IL-2 production by CsA-treated patients (96). Progress was slow, in part because the details of T lymphocyte activation were still being unraveled. However, by the late 1980s, characterization of the transcription factors/activities necessary for IL-2 gene transcription indicated that CsA was doing something in the cytoplasm of T lymphocytes (97,98). Further resolution of this problem awaited a clarification of the role of the main CsA-binding protein, cyclophilin A, in gene activation.

IV. Cyclophilin

In 1984, Handschumacher showed that 70-80% of CsA was located in the cytosol bound to a protein called cyclophilin (CyP) (99). He also showed that the immunosuppressive activities of ten cyclosporine analogs correlated with their ability to bind to CyP (100). CyP was shown to be identical to a relatively unknown enzyme, peptidyl-prolyl *cis-trans* isomerase, the activity of which was inhibited by CsA (101,102). These findings were followed by the discovery that the FK-506 binding protein (FKBP) was also a proline isomerase (103). FK-506 was a potent immunosuppressant that, although structurally unrelated to CsA, blocked T lymphocyte activation at the same stage as CsA (104,105). The finding that the activity of FKBP was inhibited by FK-506 suggested that CsA and FK-506, although chemically unrelated, acted through a common pathway by inhibiting enzymes that accelerate the rate of protein folding in proline-containing substrates.

CyP and FKBP were the first identified members of larger families of related proteins, the cyclophilins and FKBPs, respectively, that are collectively termed immunophilins for their ability to bind immunosuppressive drugs. The first CyP, now called CyP-A, is one of at least five mammalian intracellular CyP homologues (106) and these are found in a variety of tissues (107) and in different subcellular compartments (see Table 1). In addition, there are at least 25 other CyPs found in species as diverse as bacteria, slime molds, plants and birds (reviewed in (108)). The FKBP family is also large, being comprised of at least 20 members represented in an equally diverse group of organisms. All human CyPs appear to bind CsA using similar residues (109), as does a bacterial CyP (110), suggesting the tertiary structure of the CyPs has been conserved. Crystal and solution structures indicated that CsA residues 9-11 and 1-3 contact CyP (111,112).

Following the demonstration that CsA inhibited CyP isomerase activity, attempts were made to identify the biologic activities of the CyPs. CyPs from a range of species exhibit isomerase activities (113,114). While these activities were all CsA sensitive, the concentrations of CsA required were generally much higher than the concentration required to inhibit T lymphocyte activation.

Furthermore, mutagenesis studies demonstrated that isomerase activity was not necessary for CsA binding and immunosuppression (115). Finally, rapamycin, a drug structurally related to FK-506, binds to FKBP and inhibits its isomerase activity, but has a spectrum of biologic activity that is different from FK-506 and CsA (116).

Although the immunosuppressive properties of CsA and FK-506 were not linked to their ability to inhibit the isomerase activity of their respective immunophilins, there was evidence that the drug/immunophilin complexes were nonetheless important. This included demonstration that non-immunosuppressive compounds that bound to CyP or FKBP, thus preventing binding of CsA or FK-506, respectively, antagonized their immunosuppressive effects (117,118). Genetic studies also showed that cells with immunophilin defects were relatively resistant to the effects of the drugs (119,120). Finally, while the structures of free human CyP (121) and CyP bound to CsA (111,112) were very similar, the conformation of CsA in solution was very different than its conformation when associated with CyP (122,123). This suggested that the CsA-CyP complex might form a unique binding surface, permitting interactions with non-physiologic targets. This hypothesis was proven correct in 1991 when the complexes of CsA/CyP-A, CsA/CyP-C and FK-506/FKBP were shown to bind to calcineurin, an intracellular phosphatase enzyme (124,125) and that inhibition of lymphocyte activation correlated with inhibition of the calcineurin phosphatase activity (126-128).

V. Calcineurin

CN was first identified as a major calmodulin-binding protein in central nervous system tissue (129,130). Also known as protein phosphatase 2B (PP2B), it is one of the serine/threonine phosphoprotein phosphatases, the others being PP1, PP2A and PP2C (131,132). While its catalytic domain structure shares approximately 30% amino acid identity with the catalytic domains of PP1 and PP2A (133), CN has some notable functional differences. First, CN is regulated by Ca^{2+} ions and calmodulin (129,130), which itself is a Ca^{2+}-binding protein, suggesting CN activation may be mediated by environmental stimuli that initiate Ca^{2+} fluxes. A second difference was determined in a direct comparison between the four serine/threonine phosphatases in which PP1, 2A and 2C were able to measurably dephosphorylate each of the battery of 14 substrates in comparison to the three dephosphorylated by CN (134), however no predictive characteristics were identified. More recently, PP1, 2A and 2C have been shown to have histidine phosphatase activity that is not observed with CN (135). Despite this comparatively narrow substrate specificity, CN demonstrates *in vitro* nickel-dependent phosphotyrosine phosphatase activity (131,136) and weak phosphatase activity against a variety of nonprotein phosphocompounds (136,137). These activities are of unknown physiologic consequence. Overall, its narrower substrate specificity and its regulation by Ca^{2+} suggested that CN may act as a "switch" in response to extracellular stimuli, compared to other more general roles for the other phosphatases (138).

Structurally CN is a heterodimer composed of a 59-61kDa calmodulin-binding catalytic subunit called CN-A and a 19 kDa calcium-binding regulatory subunit called CN-B. Both CN-A and CN-B are encoded by multiple genes, many of which can undergo alternative splicing to yield additional variants. While CN enzyme activity is widely distributed (139,140), the different isoforms show marked differences, with, for example, CN-Aα being the predominant isoform in brain (129) while CN-Aγ is largely restricted to testis (141). Studies have attempted to identify cell-specific expression patterns (142). However, Ueki and Kincaid have demonstrated that different murine CN-B isoforms could indiscriminately associate with different yeast and mammalian CN-A subunits (143), introducing the possibility that there may be as many as 9 different holoenzyme combinations. The general characteristics of the CN-A isoforms and CN-B isoforms are discussed below.

CN-A

In mammals, CN-A isozymes (designated α, β and γ) are encoded by at least three genes, each with alternative splicing variants (Table 2). mRNA transcripts from each allele are expressed in most tissues, although Aα and Aβ are found in particularly high quantities in the brain (133,144), whereas Aγ is found predominantly in testis (141). The human Aα and Aβ isoforms are almost identical in peptide sequence (133), whereas the deduced Aγ isoform shows only 80% identity with the others (141).

Limited proteolysis studies have identified a number of distinct domains (133,145). Although most studies have been done using the CN-Aα transcript, each of the human isoforms appear to share all of the domains. These include the catalytic domain, sites for calmodulin binding and CN-B binding, a carboxy terminus autoinhibitory domain. Finally, within the first 24 residues, there is a unique stretch of 11 prolines that becomes protease sensitive upon calmodulin binding (145). This polyproline domain is absent in non-mammalian CN-A isoforms and some mammalian CN-Aα isoforms (146), and its role is undefined.

As indicated previously, the catalytic domain of CN-A is homologous to PP1 and PP2A (133) and lies, approximately, between residues 71-350 of CN-Aα. Two β sheets form a globular β sandwich which is closed at one end (147). This sandwich encompasses a hydrophobic core, with short-chain residues near the phosphatase active site near the closed end. The relatively shallow active site has been proposed to account for the ability of CN-A to recognize phosphotyrosine residues in addition to phosphoserines and phosphothreonines (147).

Overlapping slightly with the phosphatase active site is the CN-B binding domain, which is located within a central, protease resistant core of the molecule (145) between residues 350-370 of bovine CN-Aα (147,148). Crystal structure data show that this region forms a five turn amphipathic helix, the upper surface of which is complementary to the hydrophobic groove formed by CN-B (147). The complementary surfaces, the calmodulin-independent binding of CN-B to CN-A, and the coprecipitation of the subunits from resting cells suggest a stable, non-covalent interaction between CN-A and CN-B (129). Fragments from this region have been used to study the interaction between the subunits and have showed only subtle changes in conformation of CN-B upon binding CN-A (which, being larger and more rigid, is less susceptible to alteration) (148,149). However, Ca^{2+} binding by CN-B has been shown to increase the affinity of CN-A for its substrate (150), suggesting that further studies investigating the structures in the presence and absence of Ca^{2+} are needed.

Closer to the carboxy terminus of the CN-A molecule, and independent of the CN-B binding domain is a calmodulin-binding domain. Identification of this domain was made with proteolysis experiments (145) and supported by primary sequence data that revealed homology with other calmodulin binding proteins (133). Although structurally similar to CN-B, calmodulin is functionally distinct. The Ca^{2+}-dependent activation of CN by calmodulin is highly cooperative with binding of at least three Ca^{2+} sites necessary for activity (150), likely by exposing a binding domain on calmodulin. The interaction with CN-A may be further facilitated by H+ ions acting to expose the calmodulin binding surface (151). The activation of CN-A by calmodulin is accompanied by a time-dependent inactivation (151), likely to ensure that enzyme activity remains responsive to extracellular signal-driven calcium fluxes.

Adjacent to the calmodulin binding domain is the autoinhibitory tail. Studies with clostripain proteolysis demonstrated that loss of calcium dependence of CN-A was accompanied by a calmodulin-independent 10 fold increase in the phosphatase catalytic activity (145). Using synthetic peptides, Hashimoto identified a 25 residue sequence, near the carboxy terminus, that gave complete and specific inhibition of CN-A catalytic activity (152). A T lymphoma cell line that showed a lowered threshold for calcium-dependent activation was found to have a missense mutation at an aspartic acid within this autoinhibitory domain (153). These findings supported the model that this sequence serves as a functional autoinhibitory domain and suggested that the means of inhibition was by serving as a pseudosubstrate for the catalytic site that is displaced upon calmodulin binding (150).

CN-B

In contrast to the multiple genes and multiple splicing variants described for CN-A, CN-B has often been cited as one of most highly conserved protein structures in evolution (146). With detailed study, however, multiple genes, and multiple alternative splicing variants have also been identified for CN-B. As with CN-A, CN-B transcripts are found in all tissues (140). The α isoform is generally referred as the “brain” type (given its abundance in this tissue), an alternative splice form (designated CN-Bα2) is restricted to distinct developmental stages within the testis (154,155). However, mRNA transcripts of the "testis specific" CN-Bβ isoform, implicated in spermatogenesis (141), are also found in brain, lung, thymus and heart (140).

Although the cDNAs for the different CN-B isoforms predict proteins varying in size from 170 - 216 residues (154-156), there are no obvious functional differences. The primary structure of the first isolated CN-B (CN-Bα1) indicated an overall similarity to calmodulin, with 30-35% amino acid identity in the overlapping 150 residues (157). This homology rises to 54% identity within four 12 amino acid domains (referred to as "EF hand motifs"). EF hand motifs are generally associated with Ca^{2+} binding proteins. This function in CN is supported by the 4:1:1 stoichiometry of Ca^{2+}:CN-A:CN-B (129,130) and the NMR structure of CN-B (158). The global structure of CN-B is also similar to calmodulin and includes two globular domains, each containing two EF hand motifs, connected by a kinked α helix (147). Crystal structures of calmodulin binding to its ligand and CN-B binding to CN-A indicate that while both form hydrophobic central channels for ligand binding, CN-B does this in a side-by-side manner, whereas calmodulin wraps around its ligand (159).

The CN-B crystal structure helped clarify and support previous studies done to determine the CN-A binding domain (147). As indicated above, the subunits form a tight 1:1 complex, being dissociated only under harsh conditions. While a limited number of CN-A residues are involved in this association, both NMR (148) and X-ray crystal (147) structures demonstrate a large surface area of contact with CN-B, including large segments of both globular domains. This is consistent with the difficulties encountered when trying to perform mutagenesis studies of CN-B, in that each residue contributes a relatively small amount of the binding avidity, although limited success has been achieved (160).

One final structural feature of CN-B is the posttranslational addition of a myristate group on an N-terminal glycine. This is in contrast to the pattern of acetylation usually seen with other Ca^{2+}-binding proteins. Myristylation is largely irreversible and has been demonstrated to have at least three functions in other molecules (reviewed in (161)). One function is to stabilize the protein structure and another is to target the protein to membranes. The third possibility is a more dynamic version of the second and is exemplified by recoverin, a molecule that serves as a Ca^{2+} sensor in vision and, like CN-B, is a member of the EF hand superfamily. Myristylation of recoverin alters its Ca^{2+}-binding properties; Ca^{2+} binding in turn causes a conformational change that leads to the extrusion of the myristyl group, likely facilitating association of recoverin with the retinal disc membrane. Thus myristylation may permit reversible membrane targeting (162). The role of myristylation of CN-B is currently unresolved as both cytosolic and membrane- or cytoskeleton-associated forms of CN have been reported (163).

Structure/function relationships

The preceding detail of the subunit structures allows the construction of a model for CN activation. This model indicates that in resting cells, CN-A and CN-B interact non-covalently. CN-A phosphatase activity is limited due to occupation of the enzyme active site by an aspartic acid pseudosubstrate located within the autoinhibitory domain. An increase in calcium and calmodulin concentrations leads to two independent enzyme activation steps. First, calmodulin binds to CN-A and displaces the autoinhibitory domain, allowing access of other substrates to the enzyme active site. Second, Ca^{2+} binding by CN-B leads to a conformational change that increases the affinity of CN-A for some substrates. Falling Ca^{2+} and calmodulin concentrations will lead to a reversal of these events with CN-A being "turned off."

Further mechanisms of control and fine specificity have been investigated. In addition to Ca^{2+}, Ni^{2+}, Mn^{2+} and Mg^{2+} have been implicated in the activation of phosphatase activity (137,164), however precise physiologic roles have not yet been identified. Stoichiometric amounts of Zn^{2+} and Fe^{3+} were found in CN-A (165). Crystal structure data support a role for these ions in the stabilization of the enzyme active site, possibly by enhancing hydrogen bonding (147). Finally, an additional regulatory step may involve phosphorylation of CN-A by Ca^{2+}-/calmodulin-dependent kinase II (CaM kinase II) or protein kinase C (166,167). However, the precise role of phosphorylation is uncertain, as the phosphatase activities against different substrates varied by only two fold, in contrast to the 100 fold increased activity consequent to Ca^{2+} and calmodulin binding.

Drug/immunophilin interactions

By themselves, neither CsA nor FK-506 interact with CN (124) and evidence showing direct interactions between CyP or FKBP and CN has been limited to a small number of studies in yeast (168). In contrast, the data supporting a physical interaction between drug/immunophilin complexes and CN are robust. Chemical crosslinking and photoaffinity studies had demonstrated the specific interaction of CsA/CyP with CN (169,170). Mutagenesis studies identified residues of both CN-A and CN-B that affected binding of the drug/immunophilin complexes (171,172). These data suggested that the drug/immunophilin complexes bind to both CN-A and CN-B, although the autoinhibitory domain appears to play a regulatory role in CsA and FK-506 mediated inhibition of CN activity (173,174).

The recently reported X-ray crystal structure of the CN/FK-506/FKBP complex confirmed that the drug/immunophilin complex binds both CN-A and CN-B in the region where CN-A interacts with CN-B (147). CsA/CyP likely binds to CN in a similar fashion as the FK-506/FKBP dimer because: 1) drug-immunophilin complexes compete for binding to CN (124); 2) mutations in the latch region of CN-B (residues 118-124) lead to loss of inhibition by CyP-CsA (171) and in yeast, CN mutations that render them resistant to CsA also become resistant to FK506, and vice versa (172). The crystal structure of CN-A and CN-B with the FK/FKBP complex showed the drug/immunophilin complex to be 10 Angstroms from the enzyme catalytic site, suggesting the hypothesis that substrate dephosphorylation was blocked by steric hindrance of large substrates to the enzyme active site (147). This was supported by findings that drug/immunophilin complexes blocked CN activity on large but not small substrates, such as PNPP (124). This hypothesis must be reexamined, however, in light of recent findings that indicate that the drug/immunophilin complex prevents dephosphorylation by CN by inhibiting binding of CN to its substrate (NFATp - discussed below) (175).

Beyond these physical biochemical data, the functional studies indicating that inhibition of CN activity is the mechanism of CsA (and FK506) immunosuppressive action are likewise compelling. In addition to evidence cited above (126-128), the immunosuppressive activities of CsA analogues correlated with inhibition of CN activity (176). Furthermore, transfection of mammalian cells with constitutively active CN-A molecules rendered them relatively resistant to the effects of CsA (126), whereas transfection studies using a dominant negative CN-A molecule showed inhibition of NFAT transcription factor translocation and lymphocyte gene activation (177). The NFAT transcription factor family of proteins is an important target of calcineurin in lymphocytes, and preventing dephosphorylation by CN is believed to be a critical mechanism by which CsA and FK-506 prevent lymphocyte gene activation.

VI. NFAT Family Of Transcription Factors

An important product of T lymphocyte activation is the lymphocyte growth factor IL-2. It was recognized that IL-2 gene induction required the induction of a DNA binding activity that was called the nuclear factor of activated T cells (NFAT) (178). This activity was then found to have both inducible and preexisting elements (179). The inducible activity was found to be the transcription factor AP-1 (180), which itself is a protein complex composed of hetero- or homo-dimers of proteins from the Jun- and Fos-families (45,181).

The preexisting component of the NFAT complex had been shown to be present in the cytoplasm of unstimulated cells, and in the nucleus of stimulated cells and that this translocation was sensitive to both CsA and FK-506 (97,98,179,182). Candidate proteins were identified at approximately the same time by Rao and Crabtree, which they called NFATp (for preexisting) (183) and NFATc (for cytosolic) (184), respectively. These distinct proteins each have a region that is homologous to the DNA binding domain of the Rel family of transcription factors (184,185). Contained within the 464 residue Rel homology domain (RHD) of NFATp, Jain identified a 187 residue sequence that is necessary and sufficient for both DNA binding and complex formation with AP-1 proteins (186,187). Furthermore, an eight residue sequence near the amino terminus, resembling a highly conserved motif within Rel proteins that is believed to be important in DNA binding, was shown by mutational analysis to contribute to DNA binding. An NFATp recognition consensus sequence of

GGA$^{A}/_{G}$ AA has been identified in the promoter regions of a variety of cytokine genes, including IL-2,-3,-4, GM-CSF, IFN-γ and TNF-α (186-192), although a minimal sequence of GGAA may be sufficient, particularly when adjacent to an AP-1 recognition site, allowing for the formation of a stable NFAT/AP-1 complex.

Based upon the RHD, two more NFAT family members have been identified (193,194). In addition to the RHD, all four proteins have regions rich in proline and serine residues upstream of the RHD that may represent a transcription activation domain (195). In addition to these structural similarities, all NFAT proteins share the ability to bind AP-1 and DNA, and to activate IL-2 promoter sequences in transfection experiments (186,193). From the initial reports, the only notable difference appears to be tissue distribution in that NFAT3 is found predominantly in non-lymphoid cells, in contrast to the other family members (193). Characteristics of the four NFAT family members identified (so far) are shown in Table 3.

Although first isolated from a murine T cell line, NFATp is found in the thymus, spleen and circulating peripheral blood leukocytes of mice and in a variety of murine and human lymphoid cells and cell lines (196). Distribution outside the immune system appears limited to some nervous system cells (197) and human umbilical vein endothelial cells (196), although NFATp mRNA has been demonstrated in skeletal muscle and the pancreas (193). In contrast, the distribution of NFATc has not been fully elucidated. One consideration is that NFATc, unlike NFATp, is not constitutively expressed in most cells, but is inducible in lymphocytes by activation of protein kinase C or agents that increase intracellular Ca^{2+} (184). Both NFATp and NFATc were found in natural killer (NK) cells. However, while NFATp was constitutive, NFATc mRNA accumulated only after stimulation of the cells (198), suggesting that these family members can be present in the same cell, yet be regulated differently.

Regulation of the individual NFAT proteins, particularly NFATp (by virtue of being the most easily purified), has been aggressively studied. NFATp is found as a phosphoprotein in the cytoplasm of a variety of immune cells, including T cells, B cells, macrophages and mast cells (196). Stimulation of the cell leads to a biochemical change in NFATp detectable on protein immunoblots. Whereas NFATp from unstimulated cells migrates at an apparent molecular mass of 140 kDa on SDS-PAGE gels (183), cytoplasmic NFATp from stimulated cells migrates as a band or series of bands at 120 kDa, consistent with dephosphorylation (183,199). The smaller (120 kDa) dephosphorylated (?) form is found in the nucleus 2-3 minutes after stimulation (199). Because cytoplasmic NFATc/p from resting T cells was able to bind DNA and AP-1, and because CsA could prevent nuclear translocation (183,184), it was initially believed that NFATp transcriptional activity was regulated by reversible phosphorylation, with dephosphorylation by CN being permissive for nuclear entry and DNA binding. Indeed, this idea was supported by findings that *in vitro* treatment of NFATp with purified CN can result in a size reduction in NFATp similar to that seen in activated cells (200). More recently, Wesselborg demonstrated the binding of

NFATp from T cell lysates to bead immobilized CN (175), indicating a direct interaction between CN and NFATp. Another important finding from this study was that NFAT bound to CN regardless of whether it was phosphorylated, and this binding was prevented by the complex of FK-506/FKBP. Finally, Park presented data indicating that dephosphorylation of NFATp is required for full DNA- and AP-1-binding activities in human T and B cells (201). However complex the fine regulation of NFATp activity is ultimately found to be, its critical role in cytokine gene activation will continue to keep NFATp (and the other family members) a popular subject of studies investigating the mechanism of action of CsA.

VII. Goal Of This Thesis: How Does CsA Work In Patients?

How is CsA immunosuppressive in patients? With the close correlation between the inhibition of CN activity and lymphocyte gene activation, the answer should be obvious. However, examination of the *in vitro* data shows that CsA completely inhibits CN at concentrations below 100 µg/L. Trough blood levels, occurring immediately prior to the next oral dose, in CsA-treated transplant patients typically exceed 100 µg/L. As such, *in vitro* data would predict that calcium pathway-dependent lymphocyte activation in CsA-treated patients will always be completely inhibited.

Examination of the clinical literature reveals that complete inhibition of lymphocyte activation and cytokine production cannot be the mechanism of action of CsA immunosuppression. Patients on CsA can mount vigorous immune responses: they often experience rejection episodes (202) and they have relatively few complications of immunosuppression such as opportunistic infections (203); and they can be immunized against exogenous antigens (204). Patients on therapeutic CsA express T cell cytokines during rejection episodes (205) and in response to the monoclonal anti-CD3 antibody OKT3 (206,207). These data indicate that in lymphocytes of patients on CsA, either calcineurin and the calcium-dependent pathway are not blocked, or they are bypassed by CsA-resistant pathways (24).

Thus a paradox is evident: strong *in vitro* evidence identifying CsA immunosuppressive activity being mediated through inhibition of CN activity versus strong clinical evidence that the calcium-dependent pathway targeted by CsA is not completely blocked. There are at least three possible explanations. First, CsA may completely inhibit lymphocyte CN activity. If this is the case, and this is how CsA is immunosuppressive, then CN activity and NFAT activation must play a qualitative role in cytokine gene activation, such that an intact pathway is not absolutely necessary, but can amplify another more fundamental signal transduction pathway. Second, CsA may not inhibit CN activity in patients at all. This would reopen the question as to the molecular target of CsA and would appear to be an unlikely explanation of the paradox, given the considerable *in vitro* data cited above. Finally, CN inhibition may occur, but is incomplete. Incomplete inhibition may be manifest as partial inhibition at all times, or complete inhibition for only part of a normal dosing interval. Partial inhibition of CN could account for both the immunosuppressive activity of CsA as well as the immunocompetence of CsA-treated patients.

This thesis will present an argument that resolves this *in vitro/in vivo* paradox, and will present data supporting a model for the molecular mechanism of CsA immunosuppression in CsA-treated patients.

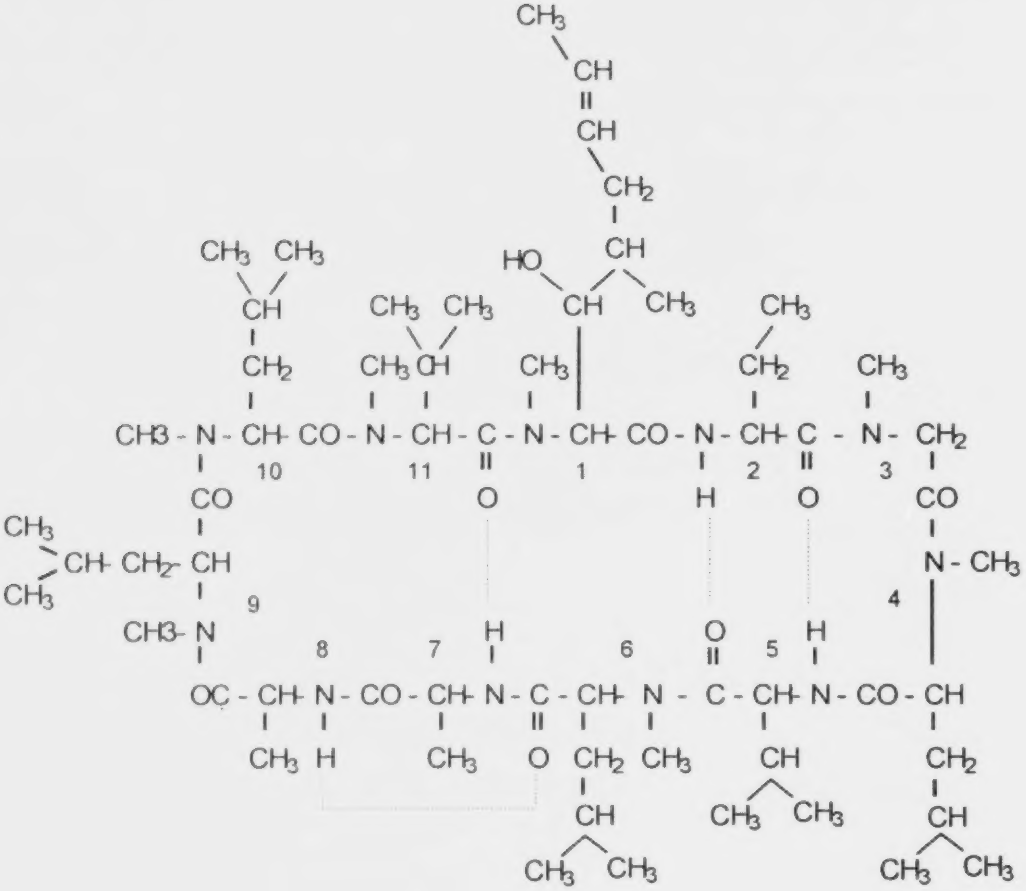


Figure 1: Structure of cyclosporine ($C_{62}H_{111}0_{12}$, M.W. 1202). Numbers indicate amino acid number and dotted lines indicate hydrogen bonds.

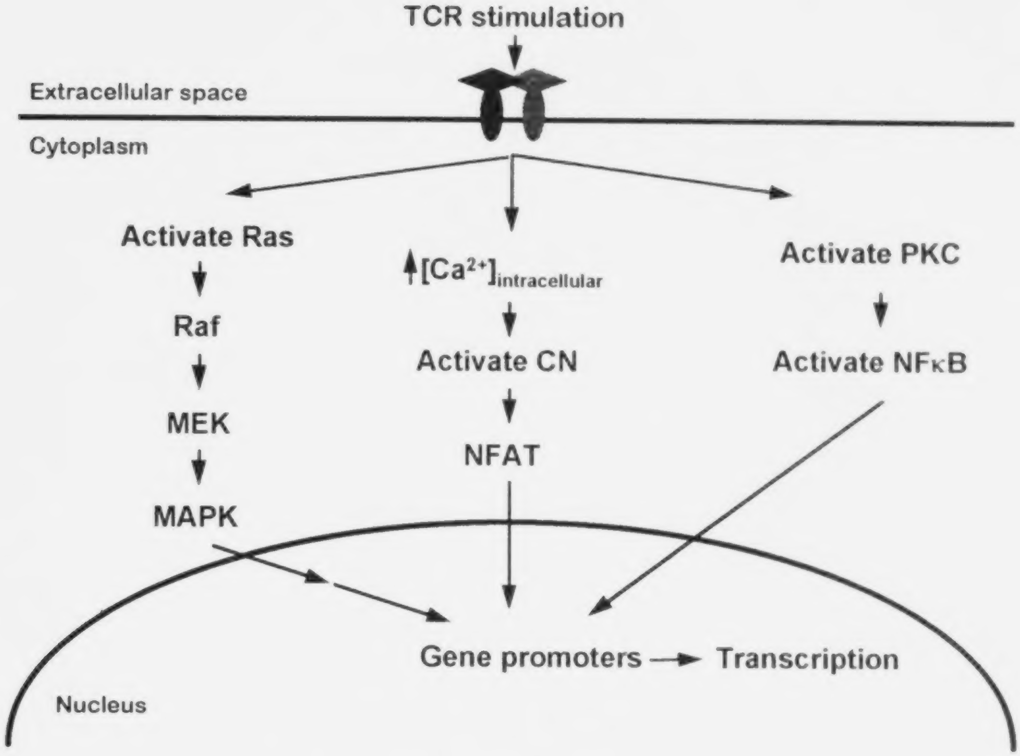


Figure 2: A schematic diagram indicating the intracellular signalling pathways believed to be important in T lymphocyte gene activation following TCR stimulation. Many details of the proximal steps following TCR stimulation have been left out. Costimulation by CD4 or CD8, and "signal 2" pathways required for T lymphocyte activation have likewise been omitted. MAPK family members are not transcription factors and must act on gene promoter indirectly.

Table 1: Characteristics of mammalian cyclophilins*

	CyP-A	CyP-B	CyP-C	CyP-D	NK-TR	CyP-40
Size (kDa)	18	21	23	18	150	40
CsA affinity (IC_{50} for rotamase inhibition)	6 nM	9 nM	6 nM	10 nM		300 nM
Inhibition of CN *in vivo*	yes	yes	no			
Tissue distribution	widespread	widespread, secreted form in breast milk	solid organs >>> T lymphocytes		NK cells	
Subcellular localization	cytoplasm	calciosome of ER	ER	mitochondria	cell surface	cytoplasm

* from (106,109,208-211)

Table 2: Comparison of the human CN-A subunit isoforms*

	Aα	Aβ	Aγ
Tissue distribution* *	brain, thymus, T cells	brain	testis
Peptide size	521 aa	514-524 aa	502 aa
Splicing variants	probably	yes	?
Polyproline domain	No	Yes	Yes
Human chromosome	4	10	8
Identity with mouse homologue	> 99%	> 99%	88%
Identity with other human CN-As	Aβ - 84% Aγ - 80%	Aγ - 81%	

* from (133,146,212-214)

** tissue distributions are often widespread, or found during distinct developmental periods; Table refers to predominant tissue

Table 3: Characteristics of human NFAT family members*

	NFAT1/NF-ATp	NF-ATc	NF-AT3	NFATx/NF-AT4
Peptide size	923-1064 aa**	716 aa	902 aa	708-1068 aa**
GenBank accession no.	U02079	U08015	L41066	U14510/L41067
Isoforms	a, b, c	?	?	a, b, c
Tissue distribution	T lymphocytes, placenta, skeletal muscle	NK cells, skeletal muscle	Placenta, lung, kidney, testis, ovary	Skeletal muscle, thymus
Activate IL-2 promoter	yes	yes	yes	yes
Direct substrate for CN	Yes	?	?	?
Identity with:	NFATc = 72% NFAT3 = 66% NFAT4 = 66%	NFAT3 = 65% NFAT4 = 69%	NFAT4 = 70%	

* from (184,193-195)

** range due to splicing variants

Chapter Two

Materials and Methods

I. **Patients:** All patients described gave informed written consent to have their blood drawn for the studies described, and the studies were approved by the relevant committees for each of the institutions involved.

II. **Mice:** Female mice aged 6-8 weeks were obtained from the Health Sciences Laboratory Animal Services (HSLAS) at the University of Alberta (BALB/c) or from Jackson Laboratories (Bar Harbor, ME) (CBA/J). The mice were maintained in the HSLAS animal colony and were kept on acidified water (1 mL of 2N HCl/litre of tap water). All experiments conformed with approved animal care protocols.

III. **Isolation of Cells and Tissues:** For studies using human materials, samples of whole blood were obtained from patients or healthy volunteers, and peripheral blood mononuclear cells (PBMC) isolated by standard Ficoll-Hypaque gradient centrifugation, which eliminates the majority of erythrocytes (RBC) and polymorphonuclear leukocytes, but with varying degrees of platelet contamination. To further reduce erythrocyte contamination, the PBMC preparation was suspended in an solution containing 0.15 M NH_4Cl, 10 mM $NaHCO_3$ and 300 μM EDTA and agitated for 3-5 minutes and the washed twice with PBS. In cases of significant platelet contamination, 2.5 units of thrombin were added to the PBMC suspension, and the resulting platelet aggregates removed by gentle centrifugation.

Isolation of purified T lymphocyte populations was performed using a negative selection column (Cellect Human T, Biotex Laboratories, Edmonton, Canada).

Enriched populations of B lymphocytes were purified by layering 25 X 10^6 PBMC on nylon wool for 30 minutes at 37°C, and recovering adherent cells by squeezing the washed wool. Monocytes were isolated by incubating 50 X 10^6 PBMC on plasma-coated Petri dishes for 90 minutes at 37°C and recovering the adherent cells. Platelets were obtained from the plasma fraction of centrifuged whole blood. Granulocytes were isolated from below the Ficoll after gradient centrifugation of PBMC suspensions. Purity of the lymphocyte and monocyte preparations was determined by flow cytometry of cell populations stained with FITC or PE-labeled monoclonal antibodies against CD14 (monocytes), CD19 (B lymphocytes) from Caltag (San Francisco, CA) and CD3 (T lymphocytes) and CD56 (NK cells) from Becton-Dickinson. RBC, platelet and granulocyte purity was assessed by microscopy.

Murine splenocytes were isolated by forcing spleen fragments through a wire mesh. Cells were washed and the erythrocytes lysed as above. In experiments using whole organs, organs were freshly harvested and immediately frozen in liquid nitrogen. For measurement of phosphatase activity, the frozen organs were weighed, then suspended in ice cold lysis buffer (see Calcineurin Assay) and homogenized. The initial homogenate was centrifuged at 3500 X g and 4°C for 10 minutes, then the supernatant was clarified by centrifugation at 15,000 X g and 4°C for 10 minutes. The supernatants were kept on ice until treated as noted.

IV. Cell lines: CCRF-CEM is a human leukemic lymphoblast cell line; CEM/VLB$_{10}$ and CEM/VLB$_{100}$ were derived from CCRF-CEM and are distinguished by being relatively (11 X and 2000 X, respectively) resistant to vinblastine compared to the parental CCRF-CEM line (215). This resistance is due to upregulation of surface P-glycoprotein molecules. Cell lines are thus designated "PGP low" (CCRF-CEM), "PGP mid" (CEM/VLB$_{10}$) and "PGP high" (CEM/VLB$_{100}$). All three lines were obtained from Dr. W. T. Beck (University of Tennessee, Memphis).

The P815 cell line is a murine mastocytoma tumor cell line (ATCC, Rockville, MD). The are maintained by passage in the peritoneum of DBA/2 mice. The ascites fluid was removed and 15-10 X 10^6 cells were injected intraperitoneally into recipient mice.

V. Culture Conditions: For *in vitro* studies, cells were cultured in a humidified (95% humidity) atmosphere at 37°C and 5% CO_2. Cell concentrations were as indicated, but generally between 1-10^6 cells/mL. Culture medium was RPMI 1640 (Gibco, Grand Island, NY), supplemented with 10% (v/v) pooled normal human serum (PHS) (NABI, Miami, FL) for studies involving freshly isolated human cells, or 10% (v/v) fetal calf serum (FCS) (Gibco) for studies involving human cell lines or murine cells, respectively. In experiments involving >16 hours of *in vitro* culture, the media were supplemented with 0.2 mM glutamine (Gibco) and an antibiotic cocktail

(Gibco) consisting of 250 μg/L amphotericin B, 100,000 units/L penicillin G and 100 mg/L streptomycin sulfate.

VI. *In vitro* stimulation: *In vitro* stimuli of PBMC (or subsets) or murine splenocytes included the calcium ionophores ionomycin (Calbiochem, La Jolla, CA) and A23187 (ICN, Mississaugo, Canada), phorbol myristate acetate (PMA; Sigma, St. Louis, MO), and the murine anti-human anti-CD3 monoclonal antibody OKT3 (Ortho, Raritan, NJ). The duration of stimulation and the concentrations were as indicated. In all cases, unstimulated control groups were treated with the solvent(s) of the stimulating agents which were: for A23187, dimethyl sulfoxide (DMSO), final concentration 0.05% (v/v); ionomycin, DMSO, final concentration 0.05% (v/v); PMA, DMSO, final concentration 1% (v/v); OKT3, double distilled water.

VII. Cyclosporine:

a) *In vitro* studies: CsA, kindly supplied by Sandoz Canada, was initially dissolved in 96% Ethanol and Tween 80 (Fisher, Fair Lawn, NJ) and a stock solution (20 mg/mL) was stored at 4°C. Stock concentrations were confirmed by measuring CsA by HPLC. Immediately before adding to culture, it was diluted to a desired concentration using medium (RPMI plus PHS or FCS). In all experiments, the concentrations of CsA solvent was kept constant in all groups ($0.35 \times 10^{-3}\%$ (v/v) ethanol and $0.14 \times 10^{-3}\%$ (v/v) Tween 80). ^{3}H-CsA tracer (final specific activity approximately 0.250 mCi/μmole) was used as indicated (kindly provided by Dr. R.W. Yatscoff, University of Alberta).

b) *In vivo* studies: For murine *in vivo* studies, CsA liquid (SIM) was diluted in olive oil and administered in 100 μL by oral gavage once daily at doses of 0 - 160 mg/kg/day, for the period of time indicated. For experiments using doses above 100 mg/kg/day, the protocols frequently called for feeding of CsA for three days, a two day rest followed by three more daily feedings. The rest period was frequently necessary because the mice did not tolerate (i.e. they died) the higher doses for prolonged periods. The cause of death was uniformly due to cachexia and GI disturbances.

For studies involving patient specimens, oral formulations included the standard formulation (SIM) (SandImmune®, Sandoz Pharmaceuticals, Basle) and a new microemulsion formulation (Neo) (Neoral ®, Sandoz). Neo was recently introduced on the strength of its bile-independent absorption, resulting in more reliable absorption. As a result, CsA blood levels in Neo-treated patients show less intra-patient and inter-patient variability (216-218). The oral formulation used (SIM or Neo) and the sample timing in relation to the most recent oral dose were as indicated.

c) CsA Levels: In murine studies, blood (500 μL) was collected in EDTA 16 hours after the previous dose and CsA whole blood levels were assessed by HPLC, as described (219), in Dr. R.W. Yatscoff's laboratory. For the determination of CsA tissue levels, organs were harvested and then homogenized in 0.9% (w/v) saline. CsA levels were measured by HPLC, and CsA content/gram tissue was calculated.

Whole blood CsA levels in clinical specimens were measured by fluorescence polarization immunoassay (Abbott TDx, Abbott Diagnostics, Abbott Park IL) in the routine clinical laboratory used by each of the respective centres.

VIII. Calcineurin (CN) activity: The assay was based on the protocol described by Fruman et al (220) and is discussed in detail in Chapter Three. This involved suspending cells in 100 μL of a lysis buffer composed of: Tris HCl pH 7.5 50 mM, DTT 1 mM, PMSF 10 mg/L, trypsin inhibitor (from soybean) 10 mg/L, leupeptin 10 mg/L, aprotinin 10 mg/L EDTA 1 mM and EGTA 0.1 mM. Cells were disrupted by three cycles of freezing in liquid nitrogen and thawing at 30°C. The lysates were centrifuged at 15,000 X g for 10 minutes and the supernatants were assayed. The assay conditions involved mixing 20 μL of supernatant with 40 μL of assay buffer containing: Tris HCl pH 8.0 20 mM, NaCl 100 mM, $MgCl_2$ 6 mM, DTT 500 μM, BSA 0.1 mg/mL $CaCl_2$ 100 μM, okadaic acid 750 nM and 7.5 μM of substrate. The substrate was a ^{32}P serine-radiolabeled 19 amino acid peptide (DLDVPIPGRFDRRV(^{32}P)SVAAE) based on a sequence from RII subunit of cAMP-dependent protein kinase. After 15 minutes at 30°C the reaction was stopped with the addition of 500 μL of a cold solution containing: KH_2PO_4 100 mM and trichloroacetic acid 5% (w/v). The free ^{32}P was collected by passing the mixture through a 0.5 mL DOWEX AG 50 X 8, 200-400 mesh column that had been prepared by washing with 1 mL NaOH 1 M, then 2 mL HCl 1 M followed by 4 mL of water. Following elution of the ^{32}P, the columns were washed once with 0.5 mL water. The eluates were collected in scintillation vials, to which scintillation fluid was added to 4 mL. The tubes were counted for three minutes in a β counter. Non-specific background activity from an assay sample composed of lysis buffer without cells plus assay buffer was determined and subtracted. For patient samples, individual patient values were compared against the mean CN activity in a group of healthy controls and CN inhibition was reported as a percentage of this control (controls defined as 100%). For *in vitro* studies, results were normalized against activity in PBMC exposed only to the solvent for CsA.

IX. Immunoblotting: After treatment, whole cell lysates were prepared by suspending T lymphocytes in lysis buffer containing 5 mM Tris pH 8.0, 25 mM NaCl, 5% sodium dodecyl sulfate (SDS), 30 mM sodium pyrophosphate, 5 mM EDTA, 2 mM PMSF, 250 μM leupeptin, 100 mg/L aprotinin and 5 mM iodoacetamide. Cell suspensions were boiled for 5 minutes, passed through a 26 gauge needle, boiled for 10 minutes then centrifuged for 40 minutes. Proteins in the supernatant were quantitated (BioRad method), separated by 6% (w/v) SDS-PAGE, transferred to nitrocellulose, then probed for NFATp using a rabbit polyclonal antibody (Upstate Biotechnology, New York, NY) that was raised in rabbits against a 464 residue recombinant peptide and is crossreactive with human NFATp (183). Bands of interest were quantitated by densitometry.

X. RNA extraction and Northern blotting: After 1 hour stimulation cells were harvested, washed with PBS and total cellular RNA extracted as described (221,222). RNA was electrophoresed on 1.5% (w/v) agarose-2.2 M formaldehyde gel using 15 μg RNA per well and transferred on nitrocellulose membrane for hybridization with ^{32}P-labeled cDNA probes. Probing was performed using a standard procedure (223). Quantitation of ^{32}P-labelled Northern blots and EMSAs (see below) was performed using a Bio-imager (Fuji BASS 1000, Fuji Photographic Company, Tokyo, Japan).

XI. Reverse transcriptase polymerase chain reaction (RT-PCR): RNA extracted from PBMC was first reverse-transcribed into IFN-γ or IL-2 cDNA using reverse transcriptase (BRL, Burlington, Canada) and oligoprimer specific for 3' end of IFN-γ or IL-2 cDNA (antisense). For a 10 μL reaction mixture, the following amounts of reagents were used: 1 μL 10 x PCR buffer, 4 μL dNTP mix (10 mM), 1 μL antisense primer (100 ng), 0.15 μL (500 units/mL) RNasin (Promega, Madison, WI), 0.25 μL (50 units) RT, 2.6 μL DEPC H_20 and 1 μL (0.01-1.0 μg) RNA. Alternatively, the reverse transcription was performed using poly-dT (200 ng/reaction) instead of a specific antisense primer. In this case, RNA concentration was increased twice as compared to specific reverse-transcription but all other parameters remained unchanged. Negative control containing all components of a mixture except RNA was included in all experiments. RT reaction was performed at 42°C for 40 - 60 minutes. For cDNA amplification, 10 μL of RT reaction mixture was combined with 40 μL of PCR reaction mixture containing 4 μL 10 x PCR buffer, 1 μL sense primer (100 ng), 0.1 μL (0.5 U) Taq DNA polymerase (BRL) and 34.9 μL DEPC-H_2O giving a total volume of 50 μL/reaction. In case of poly dT RT, specific antisense primer (100 ng/reaction) was also added to PCR mix. Amplification was performed in a Tyler thermocycler (Tyler Research Instruments, Edmonton, Canada) for 20 - 30 cycles (number of cycles depended on the initial concentration of RNA in a sample being amplified). After initial melting stage (2 minutes at 94°C), each cycle included denaturation (1 minute at 92°C), annealing of primer and fragment (30 seconds at 52°C) and extension (1 minute at 72°C). Final extension stage was at 72°C for 10 minutes.

Oligonucleotide primers for IFN-γ cDNA amplification were as follows:

5' end specific: 5' GGCTTTTCAGCTCTGCAT 3';

3' end specific: 5'GGATGCTCTTCGACCTCG 3'.

Primers for IL-2 were:

5' end specific: 5' GATGATGCTTTGACAGAAGGCTATCCATCT 3';

3' end specific: 5' CAGTGCACCTACTTCAAG 3'.

Primers were synthesized using PCR-MATE DNA synthesizer (Applied Biosystems, Mississauga, Canada). Amplified product (470 bp for IFN-γ and 390 bp for IL-2) was analyzed by ethidium bromide staining after electrophoresis on 1.5% agarose gel and by specific hybridization with ^{32}P-labeled IFN-γ or IL-2 cDNA probe after Southern blotting.

XII. Electrophorectic mobility shift assay (EMSA): Following treatment of PBMC, nuclear extracts were prepared by washing the PBMC first with PBS, then with buffer A (10 mM Hepes, pH 7.8; 15 mM KCl; 2 mM $MgCl_2$; 1 mM EDTA; 0.1 mM PMSF). The pellet was then suspended in buffer A plus NP-40 (0.2% v/v) and left on ice for 5 minutes. The cells were then centrifuged at 3000 rpm for 3 minutes, and the nuclear pellet was resuspended in 315 μL of buffer B (50 mM KCl; 0.1 mM EDTA; 1 mM DTT; 0.1 mM PMSF; 10% (v/v) glycerol) and the proteins precipitated with 15 μL of 3 M NH_4SO_4, incubated with agitation on ice for 30 minutes, then spun at 10,000 X g for 20 minutes. An equal volume of 3 M NH_4SO_4 was added to the supernatant and the mixture was incubated on ice for 20 minutes, followed by centrifugation at 13,000 X g for 30 minutes. The pellet was suspended in buffer B and protein concentrations measured.

Complementary double-stranded oligonucleotides were synthesized using PCR mate (Applied Biosystems). The sequence generated is the "P2" sequence identified by Campbell (192) as an NF-AT binding site in the human IFN-γ promoter. The sequence was:

5' GATCTAAAATTTCCAGTCCTTGA 3'.

The oligo probe was radiolabeled with ^{32}P using Klenow to fill in the ends. Competitors included:

Mutant P2 5' GGGAGGTACAAAATCGACGCCAGTCCTTGAATCCTGTGAA 3'

IL-4 "P" site 5' GATCTGTGACTGACAATCTGGTGTAACGAAAATTTCCAATGTA 3'.

Labelled probe (≈25,000 dpm) was added to the binding reaction, and 12 μg of nuclear extract was incubated with binding buffer containing 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT, 12.5% (w/v) glycerol, 50 mM KCl and 0.1% Triton-X 100. Poly dIdC (2 μg) and 5 μg BSA were added to each sample. Samples were incubated at room temperature for 30 minutes prior to running on a 6% (w/v) TBE gel for 90 minutes. The gel was dried and autoradiographed. For competition/supershift experiments, 100-200 X excess of unlabeled competitor oligos were added to the extracts and incubated for 10 minutes at room temperature prior to the addition of labelled probe. Characterisation of the binding protein(s) was carried out by determining the effect of incubating the protein:DNA complex with anti-NFAT antibodies. These were added to the binding buffer for 4 hours at 4°C prior to running the gel. The antibodies included an antimouse anti-NFATp polyclonal antibody (Upstate Biotechnology, New York, NY) that was raised in rabbits against a 464 residue recombinant peptide and is crossreactive with human NFATp (183). The other antibody was the antihuman anti-NFATc monoclonal Ab G1-D10 (courtesy of Dr. G. Crabtree, Stanford) that was raised against a 134 residue peptide that lies just amino terminal to the RHD.

XIII. Transient transfection and chloramphenicol acetyltransferase (CAT) assay: Transient transfection experiments were performed with PBMC blasts that were prepared by culturing freshly isolated PBMC in medium (with 20% v/v PHS) with 1% (w/v) PHA (Gibco) for 72 hours. Blasts were suspended at 20 X 10^6

cells/mL with 80 μg DNA, comprising a reporter gene construct containing three repeats of the CsA-sensitive "P2" sites found in the human IFN-γ gene (192) upstream of the pCATPR plasmid (Promega, Madison, WI). This plasmid has the SV40 promoter and the chloramphenicol acetyltransferase (CAT) gene. Following 10 minutes at room temperature, the blast/DNA mix was electroporated (Bio Rad, Hercules, CA) at 0.250 V and 960 μF in 250-400 μL aliquots. The samples were allowed to sit at room temperature for another 10 minutes, following which the aliquots were mixed, suspended at 1 X 10^6 cells/mL and incubated at 37°C for 1 hour prior to addition CsA. Ten minutes after addition CsA, cells were stimulated with A23187 5 μM for 48 hours.

To obtain cell extracts, cells were washed twice in PBS, once in TEN (40 mM Tris-HCl pH 7.5, 1 mM EDTA pH 8.0, 150 mM NaCl), and resuspended in 100 μL 0.25 M Tris-HCl, pH 7.5. The cells were then lysed by three cycles of freeze-thaw and the cells centrifuged at 10,000 X G for 5 minutes. The supernatant was removed and heated to 65°C for 10 minutes. CAT activity was assayed by combining 75 μL of the supernatant, 4 μL of ^{14}C-chloramphenicol (Amersham), 4 μL 5 mg/mL N-butyryl CoA (Sigma) and 42 μL 2 M Tris-HCl pH 7.5. After incubation at 37°C for 24 hours, 300 μL of xylene was added, and the samples vigorously mixed. After 3 minutes centrifugation, the top layer was removed, 100 μL Tris-HCl added, the mixture vortexed and recentrifuged. The top layer was counted in a liquid scintillation counter.

XIV. Radioactive antibody binding assay (RABA): Hybridoma cell lines producing anti-I-A^d (25-9-176SII) and anti-H-2D^d (34-4-20S) were obtained from American Type Culture Collection (Rockville, MD). mAb were purified by protein A chromatography, concentrated by Amicon ultrafiltration and radiolabeled by the Iodogen (Pierce Chemical Co., Rockford, IL) method as described (224).

Tissues of individual mice were harvested and snap frozen in liquid nitrogen. The organs were then homogenised by polytron in 1 mL PBS, centrifuged at 2000 X g in pre-weighed Sorvall tubes, washed with 10 mL PBS, and recentrifuged for 20 minutes. The supernatants were discarded and the tubes re-weighed. The pellets were polytroned again in 1 mL PBS, following which the tissue concentrations were adjusted to 20 mg/mL. Five mg of tissue was into glass tubes and centrifuged at 2000 X g for 20 minutes. The pellets were resuspended in 100 μL radiolabelled mAb in 10% (w/v) NMS (100,000 cpm) and were incubated on ice with agitation for 60 minutes. One mL PBS was added to the tube and centrifuged at 2000 X g for 20 minutes. The pellets were counted in a gamma counter, and the non-specific binding of a negative tissue was subtracted. Samples were performed in triplicate.

XV. Measurement of IFN-γ: IFN-γ in tissue culture supernatants was assessed using IFN-γ RIA kit (Centocore, Malvern, PA) with modifications for greater sensitivity as described by Woloszczuk et al (225) or by IFN-γ ELISA kit (Biosource International, Camaril, CA or Endogen, Boston, MA).

XVI. Mixed lymphocyte reaction (MLR): MLR was performed using 5 X 10^4 stimulator and 5 X 10^4 responder PBMC in a total of 0.2 mL medium in sterile 96 well plates. Stimulator cells were irradiated with 2000 rads prior to addition to culture. Sixteen to twenty four hours prior to harvesting, 2 mCi of ^{3}H-thymidine was added to each well. Cells are harvested, washed, counted and added to scintillation vials. Four mL scintillation fluid was added and vials counted.

XVII. Recovery: Red blood cells (RBC) were isolated from whole blood after centrifugation and removal of the buffy coat (used for PBMC isolation). Under recovery conditions involving RBC, CsA pre-treated PBMC were washed, resuspended at 10 X 10^6 cells/mL and diluted 1:1(v/v) with packed RBC. RBC concentrations indicate dilutions starting with concentrated RBC. Thus 1:1 indicates 1 volume PBMC (final concentration of 5 X 10^6 PBMC/mL) and 1 volume of undiluted RBC, 1:2 indicates one volume PBMC with one volume of RBC diluted one in two, etc. After recovery with RBC, PBMC were reisolated by Ficoll-Hypaque centrifugation as before. RBC contain some "contaminating" PBMC that may be included in the final sample, but due to size differences between cell lines and PBMC, we have determined the maximum contamination to be 2% of the total final cell number. Some recovery experiments utilized anti-CsA antibody (8.8 g/L), kindly provided by Dr. Max Schreier (Sandoz, Basle).

XVIII. Statistical analysis: Statistical analyses were performed using Prism software (GraphPad Software, San Diego, CA) and are described for individual experiments.

Chapter Three

Establishing the Assay and Patient Data

I. Characterizing The Activity

The first step was to determine if CN activity could be measured in freshly isolated PBMC. The conditions described by Fruman (220) were adapted to PBMC from healthy controls. In addition to CN activity, three other serine-threonine phosphatases are present in mammalian cells. These include PP1, PP2A and PP2C. The assay conditions contained okadaic acid, a compound that inhibits the activity of PP1 and PP2A without affecting the activity of CN or PP2C (131,226). PP2C, in addition to being resistant to okadaic acid, is also resistant to CsA (227). PBMC (5×10^6) in medium were exposed to CsA *in vitro* for one hour at 37°C (Figure 3). Phosphatase activity, expressed as dpm ^{32}P released, in control PBMC was reduced by 88% at CsA concentrations of 500 μg/L. The pattern of inhibition by CsA fit a sigmoidal dose-response curve with an r^2 value of 0.99, consistent with a single compartment model for the inhibition of CN by CsA. The IC_{50} was 21 μg/L, similar to previously reported CsA inhibition of CN in T lymphocyte cell lines (220). Four repeats of this experiment yielded similar results. The CsA-resistant activity was likely due to protein phosphatase 2C (PP2C), which is discussed below.

Further identification of the CsA-sensitive activity was then undertaken. Four $\times 10^6$ PBMC/group were lysed after incubation at 37°C for 30 minutes in the presence of medium alone, CsA at 1000 μg/L, FK-506 at 100 μg/L or both (Figure 4). As expected, the CsA inhibited ^{32}P release by 89%. FK-506 at 100 μg/L also inhibited ^{32}P release (Lane 3). Although total inhibition by FK-506 was somewhat less that that seen with CsA in this experiment, repeat experiments showed no difference in total phosphatase inhibition between these agents. The combination of CsA and FK-506 concentrations that each maximally inhibit ^{32}P release showed no additive effects (Lane 4), indicating that CyP and FKBP are not limiting. Performing the assay in the absence of Ca^{2+} and with EGTA 20 μM reduced ^{32}P release to the same extent as high concentrations of CsA and FK-506 (Lane 5). Finally, incubation of lysates with an anti-CN IgG antibody raised in rabbits against bovine brain CN holoenzyme (145) led to dose-dependent reduction in ^{32}P release compared to a control antibody (Lanes 6-9). The effect of the anti-CN antibody on other enzymes was not tested. Although limited availability of the anti-CN antibody prevented us from determining optimal conditions for CN inhibition, these results indicate that the CsA-sensitive serine-phosphatase activity is equally sensitive to FK-506, is Ca^{2+}-dependent and is specifically inhibited by an anti-CN antibody. Thus, the data strongly supports this activity as being due to CN.

The substrate was a (^{32}P)serine-labeled 19 amino acid (aa) peptide derived from the RII subunit of cAMP-dependent protein kinase (228). To characterize the substrate specificity for the phosphatase activity identified in PBMC, lysates of CsA-treated PBMC were prepared and the phosphatase activity measured against both the 19 aa substrate as well as a ^{32}P(threonine)-labeled 46 aa substrate derived from the sequence of protein phosphatase inhibitor-1. The 46 aa substrate was a kind gift from Dr. C.F.B. Holmes (Department of Biochemistry, University of Alberta). These experiments did not include a negative control substrate. As shown in Figure 5, activity was similar against both substrates, with IC_{50}s for CsA inhibition of 13 μg/L and 16 μg/L (p = not significant). These findings are consistent with reports showing that PPI-1 is an *in vivo* substrate for CN (229).

Finally, CsA inhibition of cytokine induction in lymphocytes has been linked to inhibition of CN. We sought supportive evidence that the CsA-sensitive activity we were measuring in PBMC was due to CN by examining the effects of CsA on the induction of steady state mRNA levels in freshly isolated PBMC. Stimulation of PBMC with a calcium ionophore (ionomycin or A23187) induces IFN-γ and IL-2 mRNA within 15 minutes, with peak expression by 30 to 60 minutes (230). To examine the effect of CsA, PBMC were stimulated with A23187 (5 μM) for one hour at 37°C, whole cell RNA was isolated, run through an agarose gel, transferred to nitrocellulose and probed with ^{32}P-labelled cDNAs for IFN-γ, IL-2 or the housekeeping gene β actin (Figure 6). As shown, CsA inhibited the induction of steady state mRNA levels for both cytokines within the same dosage range effective for inhibition of phosphatase activity.

II. Optimizing The Experimental Conditions

Having established an assay that we believe measured CN activity in PBMC, we wished to apply it to clinical specimens. To do so, we wished to standardize the assay as much as possible. This led us to a series of experiments that examined the assay conditions, the nature of the CsA-resistant background phosphatase activity and finally the variability of phosphatase activity measured between people and between experiments. Results from these investigations will be discussed in this section.

Assay conditions

We studied the effect of the number of PBMCs lysed on ^{32}P release. PBMC (10 X 10^6) were exposed to CsA solvent, CsA 10 μg/L (to model partial CN inhibition) and CsA 100 μg/L (for complete inhibition). PBMC were then lysed in 100 μL lysis buffer at concentrations of 1, 2, 4, 8 and 12 X 10^6/group and assayed in triplicate (Figure 7). ^{32}P release increased in the CsA 0 and CsA 10 groups with increasing cell number up to 12 X 10^6 cells/group, suggesting a saturation of activity (Figure 7A). One possible explanation is that, because resuspension of the pellets in this group was very difficult, lysis of the cells may have been inefficient. Excluding this last group, the increase in activity was proportional for CsA 0 and CsA 10 (r^2 = 0.88 and 0.91, respectively), such that the relative proportion of CN inhibition was the same at each cell number (Figure 7B). The amount of ^{32}P released was not linearly related to cell number, suggesting that the assay was approaching a saturation plateau. However, reducing the cell number below 10^6 resulted in CsA-sensitive activity being difficult to distinguish from non-specific background activity. Despite this drawback, these results were important for allowing us to deal with clinical specimens, in which the yield of PBMC often varied considerably.

The effects of substrate concentration and assay time were then studied. PBMC (2 X 10^6) were treated with CsA, lysed and phosphatase activity measured using conditions that reflected the standard 15 minute, 1 X substrate concentration conditions, or doubled assay time, substrate concentration or both (Figure 8). Doubling the reaction time doubled ^{32}P release in all CsA treatment groups (Group 1 versus Group 2). Doubling the substrate concentration had no significant effect on ^{32}P release (Group 1 versus Group 3). Doubling both factors had no significant effect on relative ^{32}P release among the groups (Figure 8B). Thus the results were consistent over a range of experimental conditions much broader than would normally be exercised. Finally, to facilitate the collection of clinical specimens on different days for batching the CN assay runs, we needed to establish conditions that would preserve CN activity in PBMC lysates. We established lysis buffer conditions that allowed freezing of PBMC lysates for period of at least 11 days (Figure 9). The final lysis buffer recipe was: Tris HCl pH 7.5 50 mM, DTT 1 mM, PMSF 50μg/mL, trypsin inhibitor (from soybean) 50 μg/mL, leupeptin 10 μg/mL, aprotinin 10 μg/mL EDTA 1 mM, EGTA 0.1 mM and β mercaptoethanol 0.1% (w/v).

Background activity

The assay as described so far detected both CsA-sensitive phosphatase activity, presumed due to CN, and okadaic acid- and CsA-resistant activity that is likely due to PP2C. In an experiment utilizing 5 X 10^6 PBMC/sample, okadaic acid inclusion in the assay mixture inhibited total phosphatase activity by 50% (Figure 10). However, there was no effect on the relative effect of CsA, as the IC_{50}s were 8 μg/L in the absence of okadaic acid, and 7 μg/L in its presence. The okadaic acid sensitive phosphatase activity was almost certainly due to PP1 and PP2A, which are CsA-resistant. The decision to include 200-500 nM okadaic acid in the assay buffer was based on the advantage of obtaining the lowest possible CsA-resistant background activity possible.

The remaining background, due to PP2C activity, was determined by incubating an aliquot of cells in high concentrations of *in vitro* CsA. The practical aspects of this control group led us to study the time course of CN inhibition by exposing 5 X 10^6 PBMC/mL to CsA 100 μg/L (Figure 11). The maximum degree of inhibition of CN activity was seen by 1 minute, the shortest time point measurable. These findings simplified the handling of clinical specimens by allowing us to make only a single PBMC lysate in which phosphatase activity was measured in the absence of CsA (yielding CN + PP2C activity) and in its presence (yielding PP2C activity). The difference - the CsA sensitive activity - was taken as CN activity.

Variability of CN and PP2C activity in control populations.

Finally, we tried to establish normal ranges for the CN and the okadaic acid-, CsA-resistant (PP2C?) activities in the PBMC of healthy controls. We first measured these activities in a group of ten volunteers by preparing PBMC lysates from blood drawn daily for four consecutive days (Figure 12). All assays were performed on the same day under the same conditions. The CN activity in different individuals on the same day showed coefficients of variation from 13-32%, while the coefficients of variation for PP2C activity was 29-55%. Even when the same individual was tested on consecutive days, coefficients of variation of 11-25% and 0-40% were seen for CN activity and PP2C activity, respectively. These results are based on using PBMC cell number as the input value, and may vary due to differences in contamination by non-PBMC cell types such as red blood cells (RBCs) and platelets. Attempts to quantitate protein content in the cell lysates by colorimetric (Bradford and Lowry) and optical absorbance technics were unsuccessful. We found in each case that the lysis buffer contained too many interfering substances and a protein concentration that itself was 8-10 X higher than that contributed by the lysis of 2-4 X 10^6 PBMC. Finally, attempts at using the intracellular lactate dehydrogenase (LDH) enzyme activity, as measured in the routine hospital clinical laboratory, as a measure of input were likewise frustrating. As a result, we did not establish a "normal range" for CN activity. Instead, each patient experiment involved obtaining blood samples from healthy controls that were then prepared in parallel with the clinical specimens. Each patient result was then compared to the mean of a group of control values.

III. Measurement Of CN Activity In CsA-Treated Transplant Patients

CN activity in PBMC of patients on CsA: Table 4 summarises 9 experiments measuring calcineurin activity in PBMC isolated from a total of 62 CsA-treated patients, compared to controls. CsA-sensitive phosphatase activity was reduced by 50% in patients ($p \leq 0.001$). Values for control groups vary because of the short half-life ($t_{1/2}$) of the radioisotope (^{32}P) used to label the enzyme substrate. CN activity correlated with increasing trough CsA levels (Figure 13, $r = -0.390$, $p \leq 0.01$), but not with daily CsA dose ($r = -0.236$, $p = 0.11$). For individual patients, however, calcineurin inhibition was not predicted by the CsA level.

Signal transduction: We challenged PBMC isolated from CsA-treated patients with A23187 to determine whether exposure to CsA *in vivo* blocked their ability to express IFN-γ and IL-2 mRNA. A23187 invariably induced IL-2 and IFN-γ mRNA expression in PBMC of patients, despite blood levels averaging 208 ± 45 μg/L. Representative Northern blots from 2 normals and 3 patients are shown in Figure 14A; densitometry analysis of the Northern blots from 10 patients and 4 controls confirmed that PBMC from all patients could express cytokine mRNAs (figure 14B). There was a trend toward quantitatively less specific mRNA induction in patients than in controls but this did not reach statistical significance. CsA 100μg/L added *in* *vitro* completely inhibited cytokine mRNA induction in patients and controls, whereas the CsA solvent had no effect.

CN activity in other transplant populations

CN activity was studied in other CsA-treated patient cohorts. These groups included local stable renal transplant recipients, a group of unselected cardiac transplant recipients and a group of stable pediatric renal transplant recipients. The local patients were divided into those treated with the standard formulation of CsA (SIM; SandImmune®, Sandoz Pharmaceuticals, Basle) and those taking a new microemulsion formulation (Neo; Neoral ®, Sandoz). The pediatric population was based at St. Christopher's Hospital in Philadelphia and was studied in collaboration with Drs. B. Kaiser, S. Dunn and R. Quien. For these patients, blood was drawn and PBMC lysates were prepared on site, then sent along with control lysates prepared from PBMC of healthy children to Edmonton for measurement of CN activity. The cardiac transplant population was based at St. Louis University in St. Louis, MO and was studied in collaboration with Dr. L. Miller. For these patients, samples of whole blood was drawn and sent with a group of control specimens by overnight delivery for preparation and analysis in Edmonton.

The CN activities in these groups, compared to concurrent controls, and the concomitant trough CsA blood levels, are shown in Table 5. These data from a variety of patient populations supported our initial findings that CN activity was only partially inhibited at therapeutic CsA blood levels that are higher than those required *in vitro* to completely inhibit CN activity (Figure 3). This activity is not CsA resistant as it was based on the difference in phosphatase activity in PBMC lysates analyzed in the absence and presence of *in vitro* CsA.

Kinetics of CN inhibition

Because the preceding data described abundant CN activity at trough CsA levels, it remained possible that CN was completely inhibited at the high CsA concentrations that followed a usual oral dose. To study this possibility, CN activity and CsA levels were monitored at 0 (immediately pre-dose), 1, 2, 4, 8 and 12 hours in the SIM- and Neo-treated patients shown in Table 5. The results, shown in Table 6, indicate that in both groups CN inhibition rose and fell with the rise and fall in CsA levels. However, even at peak CsA levels, complete CN inhibition was rarely seen suggesting that the CN-dependent T lymphocyte activation pathway is always intact in the majority of CsA-treated renal transplant patients or that CsA inhibition of CN is rapidly reversible.

Studying the Neo-treated patients offered us the opportunity to better understand the *in vivo* relationship between CsA blood levels and CN inhibition in circulating PBMC. As expected, peak CsA blood levels were significantly higher with Neo. Neo also led to higher CN inhibition compared to SIM at 1, 2 and 4 hours post-dose. While these differences did not achieve statistical significance, high levels of CN inhibition (>95%) were more frequent in the Neo group (22% vs. 8%, p = NS). Indeed, when we calculated the degree to which CN inhibition increased following a CsA dose, Neo led to a significantly more fractional CN inhibition at one hour (Neo = 47 ± 30%; SIM = 29 ± 35%; $p < 0.05$). However, at two and four hours, the differences were no longer significant. Finally, measurement of the area under the curve (AUC) for CsA levels has been advocated as a preferable measure of CsA effect (231). In our patients, the CsA AUC over the first 4 hours was 49% higher in the Neo group compared to SIM ($p < 0.0003$), but was accompanied by only a 5% increase in CN inhibition with Neo compared to SIM (p = NS).

The eight pediatric renal transplant recipients also had pharmacokinetic/pharmacodynamic studies performed (Figure 15). All patients were clinically stable and all were receiving SIM. CN inhibition rose and fell in concert with the CsA levels, supporting the previous findings that indicated that CsA inhibition of CN activity in PBMC occurs and recovers very quickly in patients.

CN inhibition and histopathology

Finally, the study of cardiac transplant patients (CN and CsA data shown in Table 5) allowed us to examine the relationship between CN inhibition and histopathology. These patients were randomly recruited patients followed after cardiac transplantation. In addition to blood CsA monitoring, routine clinical follow-up includes transvenous intracardiac biopsies. A standardized histology grading score indicating the absence or presence of rejection allowed us to compare CN activity with histology (Figure 16). In this group of patients there was no clear pattern of histology associated with any range of CN activity.

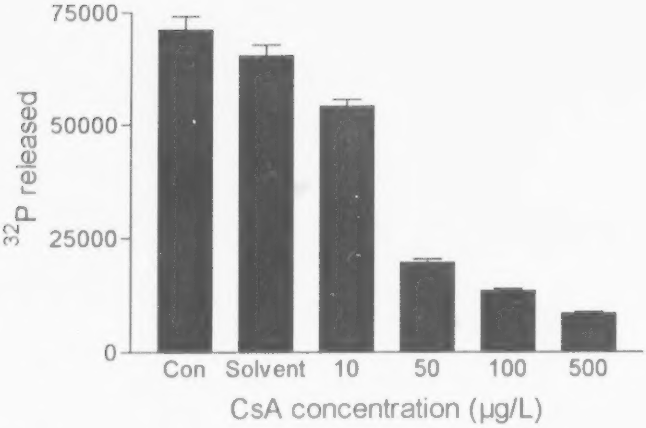


Figure 3: Inhibition of phosphatase activity in PBMC by CsA. 5 X 10^6 PBMC were incubated with medium (Con), CsA solvent (solvent) or CsA for one hour at 37°C prior to lysis and assay of release of ^{32}P from a (^{32}P)serine-labeled, 19 residue substrate. Results represent mean ± STD of triplicate samples.

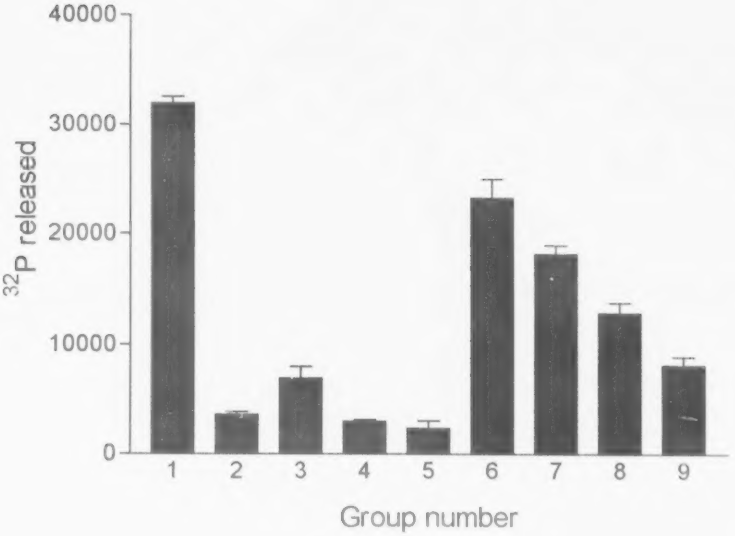


Figure 4: Effect of CsA, FK-506, EGTA and anti-CN antibody on PBMC phosphatase activity. PBMC were lysed after incubation for 30 minutes at 37°C with the following treatments: Lanes 1 and 5-9, medium; Lane 2, CsA 1000 μg/L; Lane 3, FK-506 100 μg/L; Lane 4, CsA 1000 μg/L + FK-506 100 μg/L. Some lysates were treated for 30 minutes at 37°C as follows: Lane 6, 3 μL nonspecific rabbit IgG polyclonal Ab; Lane 7, 1 μL rabbit anti-bovine brain CN IgG (αCN); Lane 8, 2 μL αCN; Lane 9, 3 μL αCN. Lysates were then assayed for phosphatase activity as before, except Lane 5 in which the assay buffer contained EGTA 20 μM instead of Ca^{2+}. Results represent mean ± STD of triplicate samples.

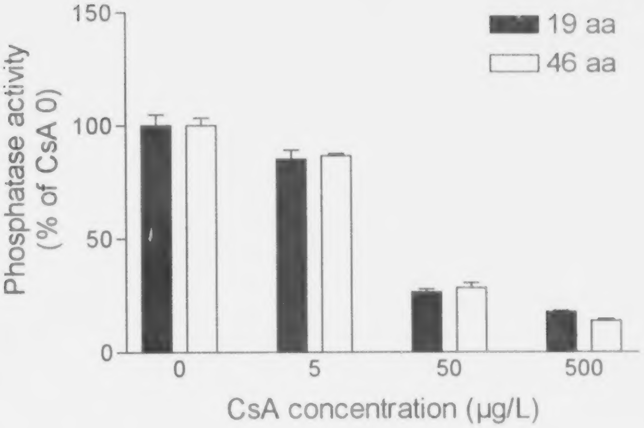


Figure 5: CsA-sensitive phosphatase activity against two peptide substrates. PBMC were incubated with CsA, lysed and phosphatase activity assayed against a (^{32}P)-serine labeled 19 residue substrate from the RII subunit of cAMP-dependent protein kinase and a (^{32}P)-threonine labeled 46 residue substrate from phosphatase inhibitor 1. Activity was normalized against CsA = 0. Results represent mean ± STD of triplicate samples.

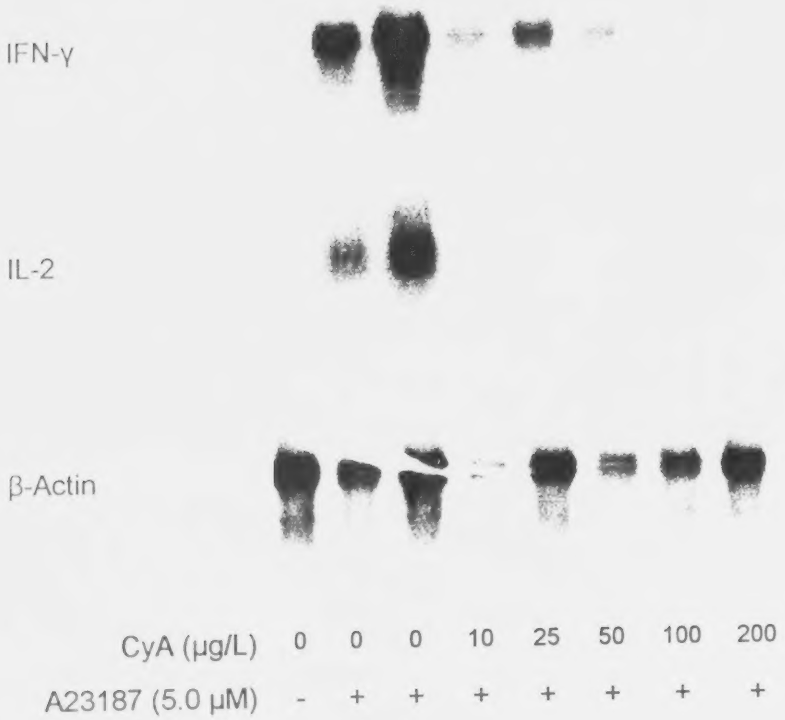
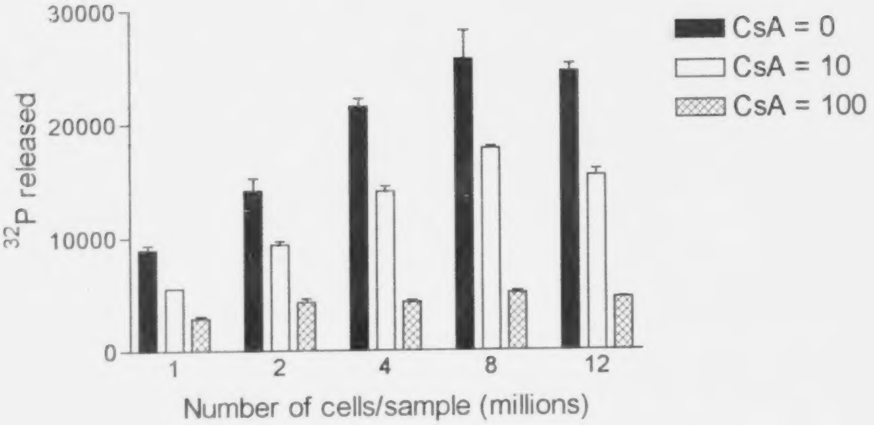

IFN-γ
IL-2
β-Actin
CyA (μg/L) 0 0 0 10 25 50 100 200
A23187 (5.0 μM) - + + + + + + +

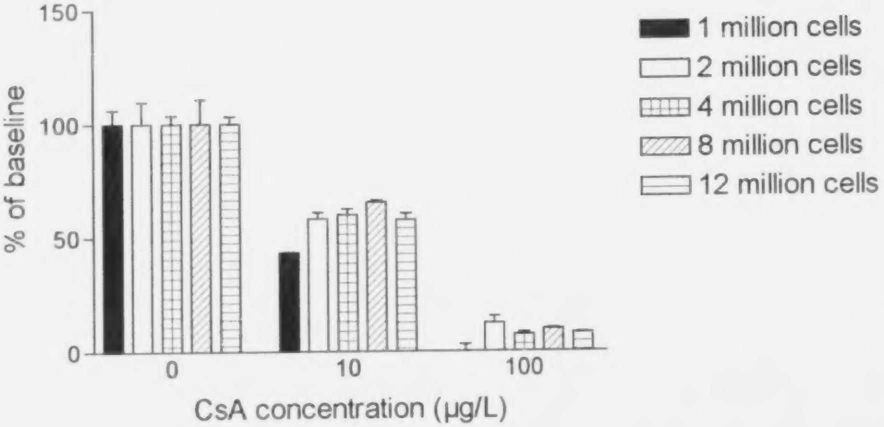
Figure 6: The effect of CsA on cytokine induction in stimulated PBMC. PBMC were incubated at 1 X 106/mL in CsA for 30 minutes at 37 C, then stimulated for one hour by A23187 5 μM. Cells were harvested, whole cell RNA extracted, electrophoresed on an agarose gel and transferred to nitrocellulose. The blot was probed with ^{32}P-labelled cDNA sequences complementary for IFN-γ, IL-2 and the β-actin genes and autoradiographed.

Figure 7: Effect of different cell numbers on phosphatase activity. PBMC were exposed to CsA 0 (solvent only), CsA 10 μg/L or CsA 100 μg/L and lysed in 100 μL lysis buffer at 1, 2, 4, 8 or 12 X 10^6 cells/sample. Assays were performed and show absolute ^{32}P release (A) or relative ^{32}P release in which the mean of the CsA 0 group was considered 100% activity and CsA 100 in the 1 X 10^6 cell group was considered complete CN inhibition (B). Each bar represents the mean ± STD of triplicate samples.

A)


30000
20000
10000
0
^{32}P released
1
2
4
8
12
Number of cells/sample (millions)
CsA = 0
CsA = 10
CsA = 100

B)


150
100
50
0
% of baseline
0
10
100
CsA concentration (µg/L)
1 million cells
2 million cells
4 million cells
8 million cells
12 million cells

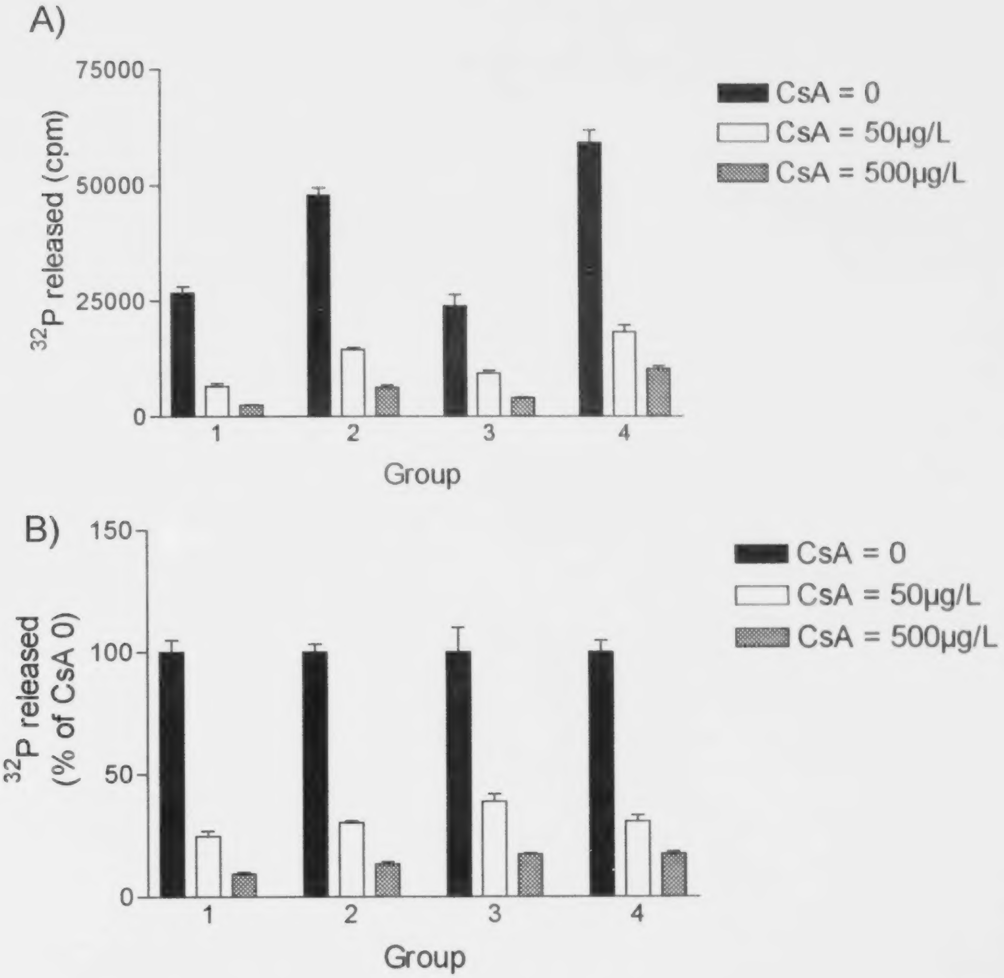


Figure 8: Effect of doubling substrate concentration and/or reaction time. PBMC (2 X 10^6) were exposed to CsA, then assayed using the following conditions: Group 1: 15 minutes, 1 X substrate; Group 2: 30 minutes, 1 X substrate; Group 3: 15 minutes, 2 X substrate; Group 4: 30 minutes, 2 X substrate. Results show raw data (A) and data normalized against CsA = 0 within each group (B) as mean ± STD of triplicate samples.

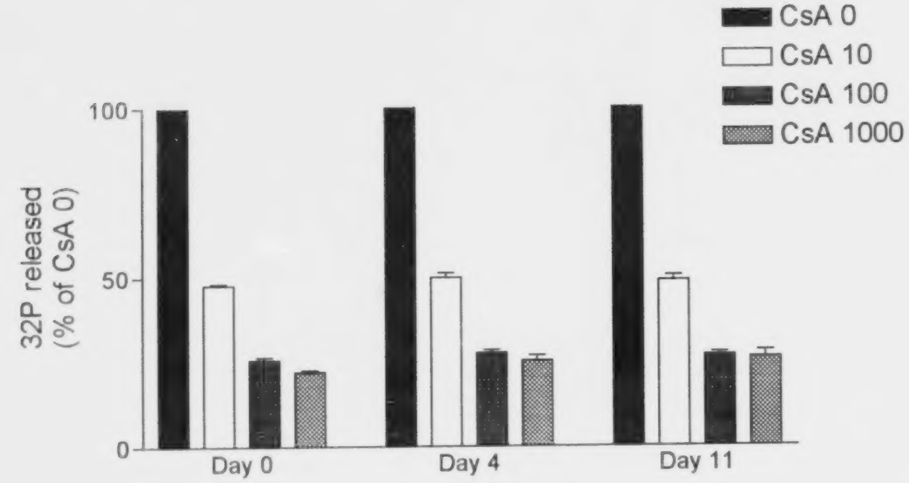


Figure 9: Effect of storing frozen cell lysates on phosphatase activity. PBMC were exposed to CsA and suspended in lysis buffer at 3 X 10^6 cells/sample. Some samples were lysed immediately and assayed for ^{32}P release. Other aliquots were frozen in liquid nitrogen until day 4 or day 11, at which time the remainder of the freeze/thaw lysis procedure was finished. The same substrate was used and activity has been normalized for maximum activity in the CsA 0 group. Results represent mean ± STD of triplicate samples.

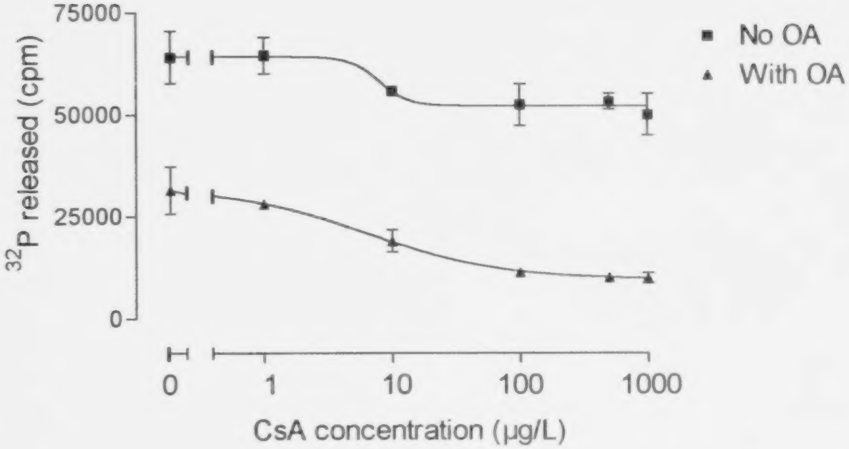


Figure 10: Phosphatase activity in the presence and absence of okadaic acid. Phosphatase assays were performed on 5 X 10^6 CsA-treated PBMC/sample without or with okadaic acid 500 nM final concentration added to the assay buffer. The IC_{50}s for the CsA sensitive component of the phosphatase activity were: without okadaic acid, 8 μg/L; with okadaic acid, 7 μg/L. Results represent mean ± STD, n = 3 samples/point. The lines represent computer-generated curves when the data were fit to sigmoidal dose-response relationships.

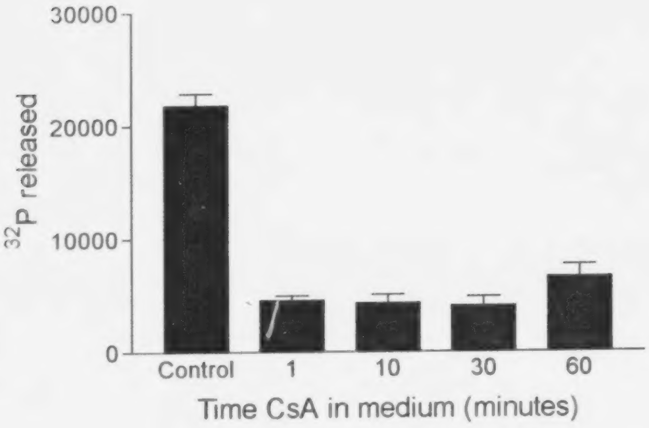


Figure 11: The time course of CsA inhibition of PBMC CN *in vitro*. PBMC (5 X 10^6) were exposed to CsA 100 μg/L for the time indicated, centrifuged, lysed and phosphatase activity measured. Control cells had CsA solvent added for one hour. Bars represent mean ± STD of triplicate samples.

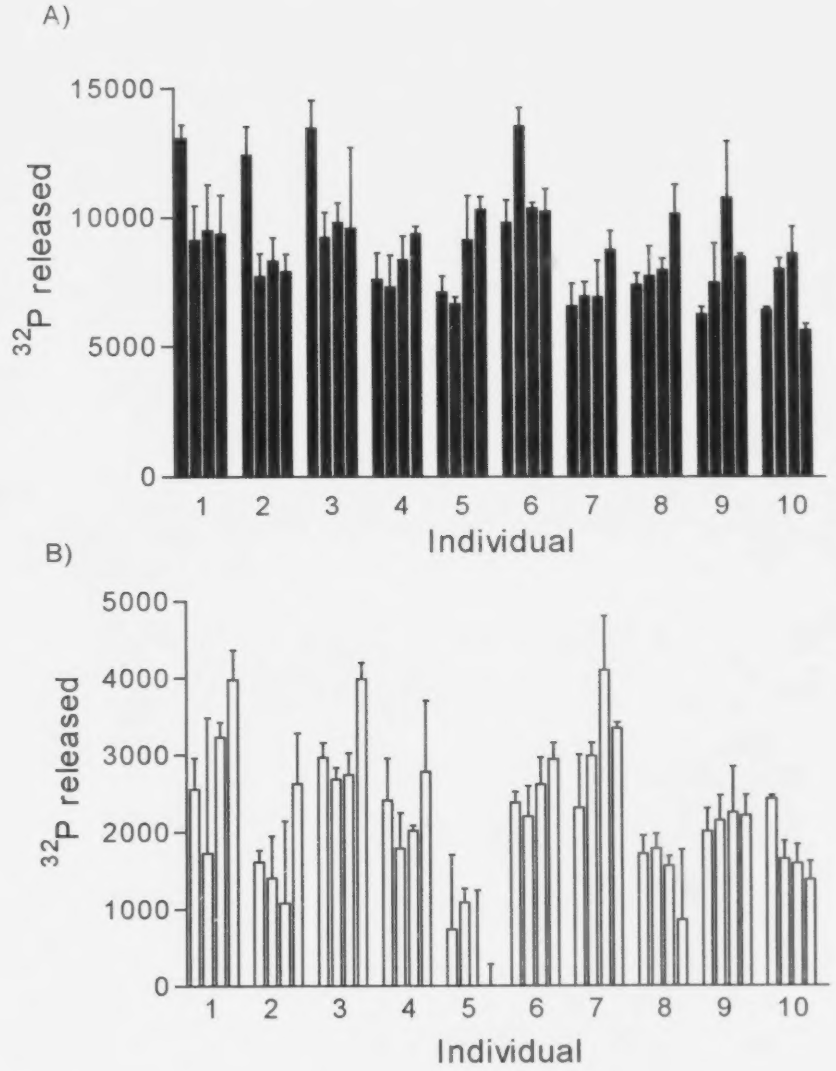


Figure 12: Variability of CsA-sensitive and -resistant phosphatase activity within and between controls. Ten volunteers had blood drawn on four consecutive days. PBMC were isolated and tested for CsA-sensitive (solid bars, A) and CsA-resistant (open bars, B) phosphatase activity. Results shown represent mean ± STD of triplicate samples on each of (from left to right) Day 1, 2, 3 and 4 for each individual.

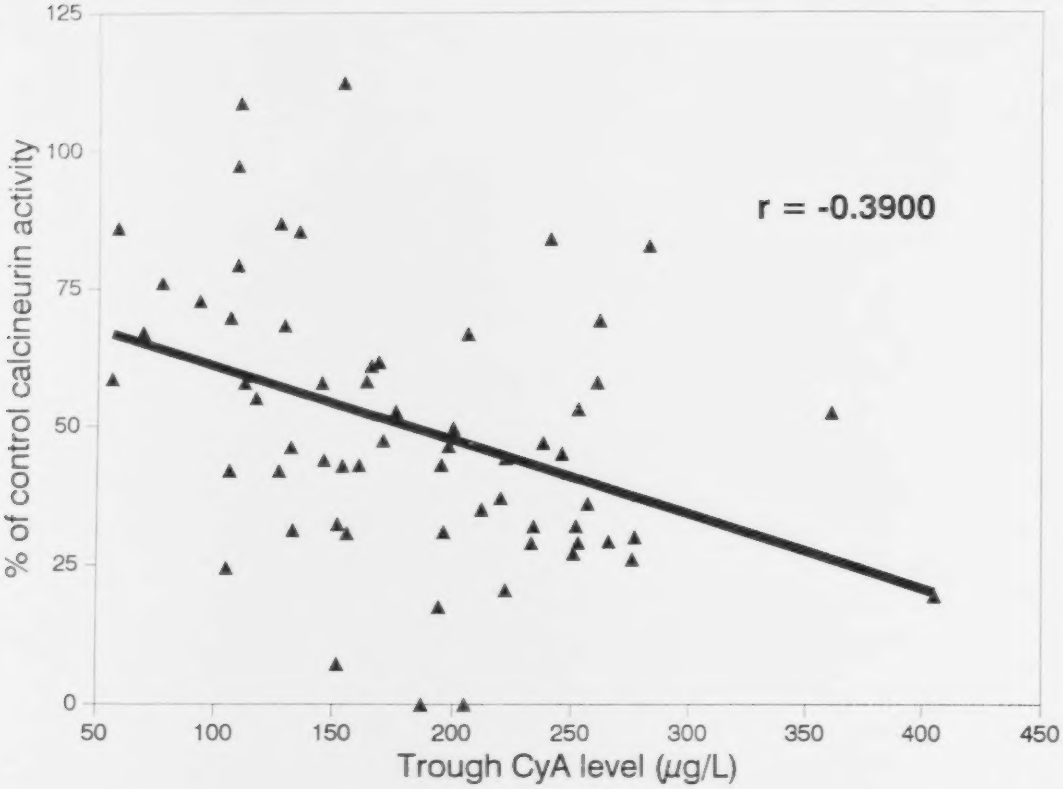
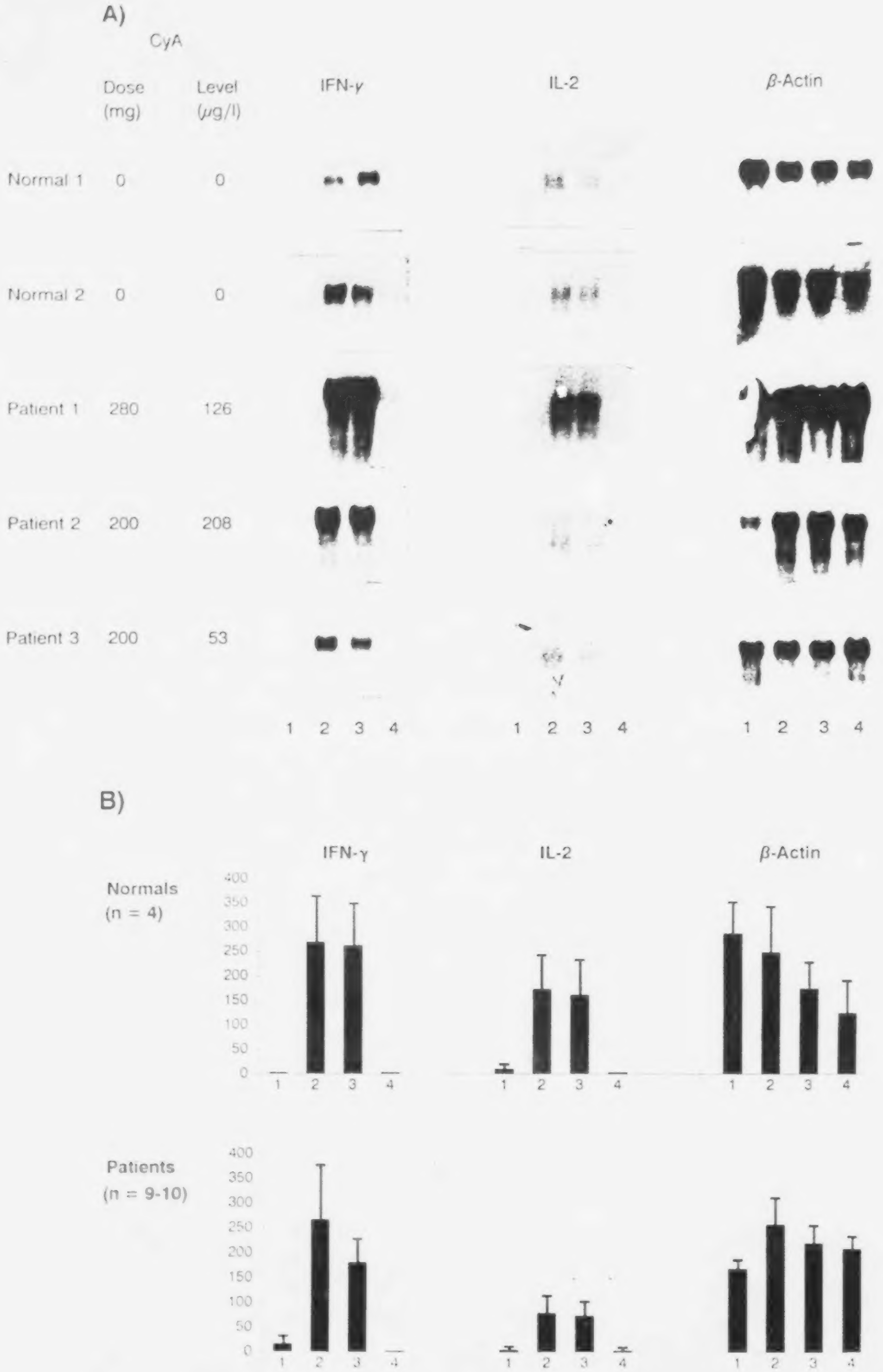


Figure 13: Comparison of calcineurin activity with trough CsA level. CN activity (as a percentage of control) plotted against trough CsA blood level in 62 renal transplant patients shows significant negative linear correlation (r = -0.390, $p \leq 0.01$).

Figure 14: Stimulation of cytokine message in CsA-treated patients. PBMC isolated from normal individuals and CsA-treated renal transplant recipients were exposed to CsA for 1 hour, then stimulated by A23187 5.0 μM for 1 hour. RNA was isolated and Northern blotting performed. Blots were probed with ^{32}P-labelled cDNA probes complementary for IFN-γ, IL-2 and β-actin (as a loading control) mRNA. Groups: 1 = unstimulated (A23187 solvent); 2 = A23187; 3 = A23187 + CsA solvent; 4 = A23187 + CsA (100 μg/L).

A) Representative Northern blots from two normal controls and three patients shown with CsA dose and trough blood levels, demonstrate no inhibition by *in vivo* CsA, but complete suppression by *in vitro* CsA.

B) Densitometry of Northern blots from controls (4) and patients (9-10). Mean CsA level for patients = 208 ± 45 μg/L. Shown in arbitrary units, mean ± SEM.


A)
CyA
Dose (mg)
Level (μg/l)
IFN-γ
IL-2
β-Actin
Normal 1
0
0
Normal 2
0
0
Patient 1
280
126
Patient 2
200
208
Patient 3
200
53
1
2
3
4
B)
IFN-γ
IL-2
β-Actin
Normals (n = 4)
Patients (n = 9-10)
400
350
300
250
200
150
100
50
0

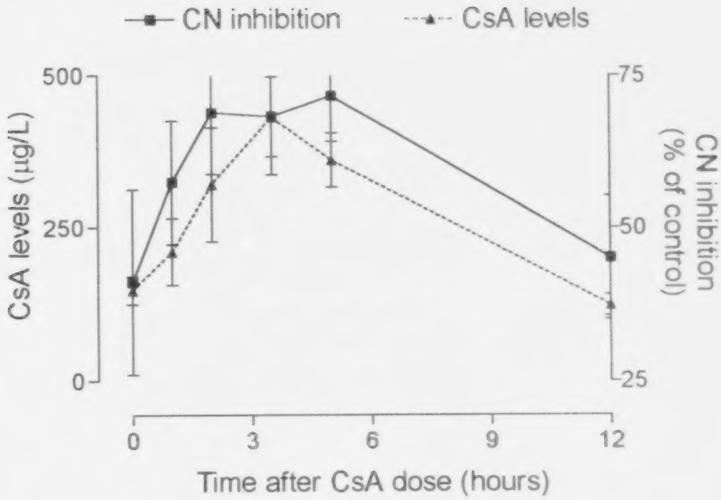


Figure 15: Pharmacokinetic/pharmacodynamic relationship between CsA levels and CN inhibition in eight pediatric renal transplant recipients after an oral dose of CsA. The patients were on twice daily maintenance CsA (SIM) therapy. Blood was drawn immediately prior to and at 1, 3.5, 5 and 12 hours following a usual morning dose of CsA. CsA levels were determined locally and PBMC lysates were prepared and sent, with pediatric control specimens, for measurement of CN activity. Each point represents the mean ± STD of the eight patients at each time point.

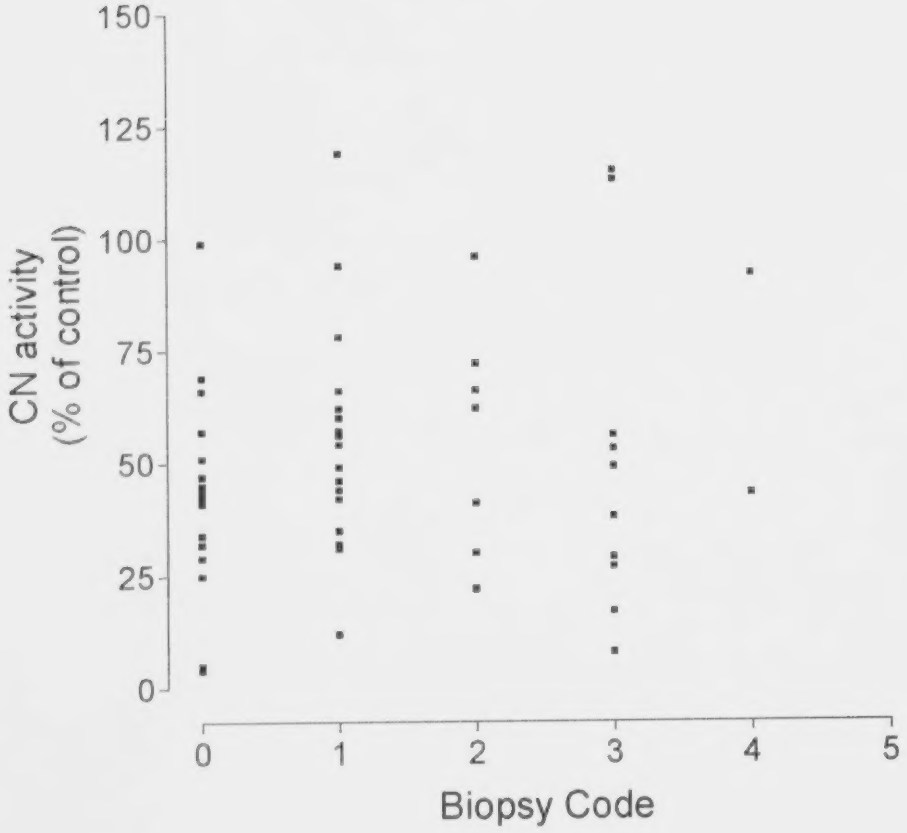


Figure 16: CN activity and pathology grades in CsA-treated cardiac transplant patients. Biopsies of 58 CsA-treated cardiac transplant recipients were read by a pathologist, following which the rejection grade was assigned a numerical value from 0 (no rejection) to 5 (severe rejection). These results are plotted against the concomitant CN activity.

[*] CN activity in PBMC reported as mean CPM (x 10^{-3}) ± STD of (^{32}P)-phosphate released.

[‡] Unpaired *t* test comparing patient group versus control group: [†] - $p \leq 0.05$; [¶] - $p \leq 0.01$; [**] - $p \leq 0.0001$

[§] Mean ± STD of trough CsA level at time of CN activity measurement

Table 4. CN activity in CsA-treated renal transplant patients is reduced compared to untreated controls.

Exp. No.		Controls (n)	Patients (n)	% Reduction[‡]
1	CN activity	23.5 ± 11.5 (3)	10.4 ± 6.3 (11)	56[¶]
	CsA level (μg/L)[§]	-	175 ± 67	
2	CN activity	43.4 ± 6.5 (4)	21.1 ± 7.2 (5)	51[¶]
	CsA level (μg/L)	-	232 ± 96	
3	CN activity	29.9 ± 6.5 (4)	15.5 ± 5.3 (10)	48[¶]
	CsA level (μg/L)	-	195 ± 92	
4	CN activity	16.9 ± 5.1 (4)	9.5 ± 4.4 (12)	44[¶]
	CsA level (μg/L)	-	161 ± 54	
5	CN activity	15.7 ± 4.8 (4)	8.7 ± 4.4 (10)	45[|]
	CsA level (μg/L)	-	151 ± 48	
6	CN activity	20.4 ± 4.1 (3)	8.8 ± 6.6 (4)	57[|]
	CsA level (μg/L)	-	178 ± 43	
7	CN activity	8.0 ± 3.2 (4)	3.2 ± 0.6 (2)	60
	CsA level (μg/L)	-	245 ± 7	
8	CN activity	28.4 ± 1.1 (3)	14.3 ± 5.5 (4)	50[¶]
	CsA level (μg/L)	-	216 ± 42	
9	CN activity	12.6 ± 0.8 (3)	4.8 ± 0.7 (4)	62[**]
	CsA level (μg/L)	-	211 ± 42	
Total	CN activity	22.2 ± 5.7 (32)	11.2 ± 5.3 (62)	50[**]
	CsA level (μg/L)	-	180 ± 65	

Table 5 Comparison of trough CsA levels and PBMC CN activity in groups of organ transplant recipients.

Population	N	CsA level (μg/L)	CN activity (% of control)
Edmonton (kidneys)*	58	188 ± 115	51 ± 27
Edmonton (kidneys)**	18	162 ± 45	47 ± 44
St. Louis (hearts)	58	172 ± 134	63 ± 92
Philadelphia (pediatric kidneys)	8	148 ± 53	59 ± 43

* SIM treated patients

** Neo treated patients

Table 6: Changes in CsA level and CN inhibition following oral CsA in patients receiving SIM or Neo *

		Time after dose (hours)			
		0 (predose)	1	2	4
CsA level (µg/L)	SIM§	190 ± 124	484 ± 311**	499 ± 280**	387 ± 216
	Neo§	162 ± 45	873 ± 489	787 ± 262	418 ± 101
CN (% inhibition)	SIM	52 ± 27	69 ± 19	69 ± 27	63 ± 30
	Neo	47 ± 44	74 ± 75	75 ± 22	67 ± 25

§ for SIM, n = 50; for Neo, n = 18

*values shown as mean ± STD

** $p < 0.0005$ compared to Neo at the same time point.

Chapter Four

Recovery

and

Tissue Distribution

The paradox of complete inhibition of CN by CsA *in vitro* but incomplete inhibition of lymphocyte activation in CsA-treated patients remained consistent with the clinical data shown in Chapter Three. CN in PBMC of CsA-treated patients was inhibited, but to a lesser degree than occurred in PBMC exposed to CsA *in vitro*. These results raised two questions that will be addressed in this chapter. First, was there a technical reason(s) for the relatively reduced CN inhibition seen in patient PBMC? To examine this possibility, we tested the following hypothesis: during the time required to separate and prepare PBMC from patient whole blood samples, there is recovery of CN activity in the PBMC. The second hypothesis tested was: the degree of CN inhibition in heterogeneous PBMC populations accurately reflects the degree of CN inhibition in lymphocytes.

I. Is There Recovery?

To address the possibility of CN recovery during the preparation of patient specimens, control PBMC were exposed to CsA 200 μg/L or solvent *in vitro*. CN activity and cytokine gene induction were measured before and after the same isolation sequence used for patient samples ("Ficoll"); a "no Ficoll" group was included as a control for separation time. As shown in Figure 17, the degree of inhibition of CN activity and IFN-γ gene induction by CsA was not affected by a typical isolation procedure. Because 200 μg/L of CsA may overestimate the exposure of PBMC in patients, we then assessed recovery of CN in control PBMC exposed to a range of CsA, both before and after washing and resuspending the cells in CsA-free medium (Table 7). As shown, recovery of CN activity was poor, although with some variability, even after 24 hours. The degree of recovery of CN activity was 0-20% following exposure to CsA concentrations of 10 µg/L. Even at CsA 1 µg/L, maximum recovery was only 50% after 24 hours. Thus *in vitro* CsA inhibition of CN activity has a very rapid "on" rate (Figure 11), but a very slow "off" rate.

If recovery of CN requires the efflux of CsA from the PBMC, a possible explanation for the limited recovery observed was that the culture medium contained an insufficient number of extracellular CsA binding sites to prevent immediate reentry of CsA back into the PBMC. To examine the effect of potential extracellular CsA binding sites, we tested whether the addition of red blood cells (RBC) to the recovery medium would affect recovery of CN activity in cells pretreated with CsA 100 µg/L. We also tested whether recovery was dependent on protein synthesis using the protein synthesis inhibitor cycloheximide (50 µg/mL). Prior to measurement of CN activity, the RBC were removed by Ficoll centrifugation. After 4 hours, there was 1% recovery of CN activity in medium alone (Figure 18). The addition of RBC increased the recovery of CN activity in a dose dependent manner, to a maximum of 50%. Recovery was not affected by cycloheximide.

To investigate the functional consequences of CN recovery, we studied the IFN-γ secretion by PBMC stimulated by either calcium ionophore (A23187) or anti-CD3 mAb (OKT3). As with CN activity, exposure of PBMC to CsA exposed to CsA, washed and resuspended in CsA-free medium, the recovery of production of IFN-γ by cells pretreated with CsA 100 μg/L or 200 μg/L was limited in culture medium alone with a range of 0 to 30% recovery at times up to 19 hours (Table 8). We then determined if recovery of IFN-γ production correlated with recovery of CN activity. PBMC were treated with CsA 100 μg/L, washed and incubated with increasing dilutions of RBC for 4 hours (Figure 19). RBC were removed and PBMC were lysed for measurement of CN activity, or suspended in medium and simulated overnight with OKT3 10 ng/mL and supernatant then assayed for IFN-γ. As shown, recovery of IFN-γ production mirrored the recovery of CN activity, indicating that recovery of CN activity has immediate functional consequences.

To characterize the movement of CsA in and out of PBMC, and examine the effect of temperature on this movement, ^{3}H-CsA (100 μg/L) treated PBMC were washed and incubated with medium or RBC for two hours at either 37° C or 4° C. While some recovery (8%, $p \leq 0.005$ compared to baseline, Figure 20) occurred in culture medium alone at 37° C, it was markedly enhanced in the presence of RBC (54%, $p \leq 0.0001$). No recovery was seen at 4° C, even in the presence of RBC (p = n.s. for both groups compared to baseline). As shown, the concentration of ^{3}H-CsA in PBMC varied inversely with CN activity.

Role of P-glycoprotein:

The temperature dependence of CsA efflux and CN recovery suggested that CsA is transported out of cells by an energy-dependent mechanism. P-glycoprotein (PGP), the product of the *mdr*1 gene, transports hydrophobic drugs, including CsA (232), out of cells. We tested the effect of CsA on CN activity in two human T cell leukemia lines that differ only in PGP expression ("PGP-low" versus "PGP-high", Figure 21). CsA 100 μg/L inhibited CN activity less in PGP-high cells than PGP-low cells ($p \leq 0.002$). After two hours in culture medium alone (no RBC), PGP-high cells showed recovery of 58% of CN activity compared to only 8% in the PGP-low cells ($p \leq 0.0005$).

To study the minimum time necessary for recovery of CN activity, PGP high cells were treated with CsA, washed and suspended in cold (4°C) medium at 10×10^6 cells/mL. They were brought to 37°C by 10 X dilution with warm (37°C) medium and incubated in a 37°C water bath for the times indicated (Figure 22). The rate of recovery for cells treated with CsA 100 or 250 μg/L was initially rapid, with significant recovery seen within 2 minutes ($p \leq 0.05$ for both), with the final degree of recovery usually seen by 5 minutes. Surprisingly, recovery was incomplete in the CsA 250 and 1000 μg/L groups.

We sought to confirm the role of PGP in the rapid recovery of PGP-high cells by specifically inhibiting PGP transport of CsA. Verapamil and quinidine (both from Sigma) were chosen as they have been shown to inhibit PGP transport of CsA (232). To confirm effective inhibition of PGP transport, PGP-high cells were incubated overnight with verapamil or quinidine (both at 100 μM, based on (232)) in vinblastine-containing medium. This resulted in greater than 90% cell death (by dye exclusion) by both agents, indicating effective inhibition of PGP transport of vinblastine.

The effect of blockade of PGP transport on the recovery of cells from the effects of CsA by incubating PGP-high cells with verapamil at 100 μM or quinidine at 100 μM in vinblastine-free medium prior to CsA exposure (Figure 23). Preincubation with both agents led to a greater degree of CN activity inhibition by CsA 100 μg/L ($p \leq 0.0005$). Recovery of CN activity was completely prevented by either agent when cells were resuspended in CsA-free medium.

Having established that PGP blockade prevented recovery of PGP-high cells in medium alone, we then determined if PGP blockade affected recovery in the presence of RBC and anti-CsA Ab, as is required for PBMC recovery. To test this, verapamil pretreated PGP-high cells were exposed to CsA 100 μg/L, washed and resuspended (Figure 24). The addition of anti-CsA Ab facilitated 17% recovery ($p \leq 0.05$ compared to medium alone). When RBC were present in the recovery medium, recovery was 71% ($p \leq 0.0005$ compared to either medium alone or anti-CsA Ab). Recovery in the presence of PGP blockade thus indicates a separate, PGP-independent mechanism for CsA efflux.

To determine if PGP activity was an important factor in the effect of CsA on PBMC, we measured the effect of PGP blockade by verapamil (100 μM) on CsA inhibition of CN in PBMC (Figure 25). Over a wide CsA concentration range, CsA inhibition of CN activity was not altered in PBMC pretreated with verapamil 100 μM compared to PBMC exposed to verapamil solvent. Furthermore, in PBMC exposed to CsA 100 μg/L, washed and resuspended in CsA-free medium (Table 9), RBC or anti-CsA antibody assisted recovery of CN activity was unaffected by verapamil pretreatment, confirming the existence of a PGP-independent efflux mechanism.

Effect of whole blood on CsA entry into PBMC

Since untreated RBC were capable of effecting CN recovery in CsA-treated PBMC, we hypothesized that RBC, or other whole blood components, by competing for CsA binding, may account for the 10-20X higher IC_{50} for CN inhibition found in patients compared to PBMC treated in culture medium *in vitro*. To test this, we exposed PBMC suspended in culture medium (CM) or whole blood (WB) to CsA. After 30 minutes at 37°C, PBMC in CM were placed at 4°C while the whole blood was layered on Ficoll. After removal of the PBMC rich layer, both CM and WB groups were washed, counted, lysed and CN activity was measured. As shown in Figure 26, there was concentration-dependent inhibition of CN activity by CsA in both CM and WB groups. The IC_{50}s for inhibition of CN activity by CsA were: CM = 2 μg/L (95% confidence interval

[C.I.] = 1-10 μg/L); WB = 102 μg/L (95% C.I. = 42-245 μg/L). Thus, there was significantly more effect on CN activity of PBMC suspended in CM compared to those still in WB ($p \leq 0.005$ using unpaired Student's t test).

Similar results were seen for CsA inhibition of OKT3-induced IFN-γ synthesis (Figure 27). While OKT3 stimulated PBMC to produce abundant IFN-γ (unstimulated cells made no IFN-γ), there was concentration-dependent inhibition by CsA in both groups, with the following IC_{50}s: CM = 18 μg/L (95% CI = 12-28 μg/L); WB = 690 μg/L (95% C.I. = 73-6454 μg/L). Again, PBMC in WB had significantly less inhibition than PBMC suspended in CM ($p \leq 0.005$). The higher IC_{50} values observed for IFN-γ compared to CN activity may reflect the incubation time (18 hours) in CsA-free medium required for the IFN-γ assay, during which partial recovery from the effects of CsA may have occurred. However, note that the relative increase in IC_{50} in the WB group compared to the CM group was similar (approximately 40-50X) for both CN activity and IFN-γ production.

Finally, to confirm that the reduced effect of CsA on PBMC in WB was a reflection of reduced CsA accumulation, we added ^{3}H-CsA to PBMC in CM or WB, with and without the presence of unlabelled CsA (Figure 28). At concentrations of unlabelled CsA from 0 - 100 μg/L, there was 8.5 - 12.2 fold more ^{3}H-CsA in PBMC exposed in CM compared to PBMC exposed in WB ($p \leq 0.005$ at all points). This ratio fell to 4.6 at a CsA concentration of 10,000 μg/L ($p \leq 0.01$), presumably due to saturation of binding sites in the PBMC in WB.

Thus typical *in vitro* conditions overestimated the CsA effect on leukocytes by an order of magnitude. This correlated with the findings that approximately 10 fold more ^{3}H-CsA accumulated in PBMC suspended in CM compared to those in WB. While complete inhibition of CN activity and cytokine induction is often seen at *in vitro* CsA concentrations of 100 µg/L, even peak CsA levels in patients following a single dose are rarely accompanied by complete inhibition of CN activity (Table 6 and Figure 15). Thus the concentration of CsA in whole blood, even when added *in vitro*, provides, at best, only an indirect measure of the effect of CsA immunosuppression at the level of the leukocyte.

II. Are We Measuring CsA-Sensitive Activity In The Right Cells?

CN activity in purified circulating human cell types

To address this question, we prepared enriched populations of each of the different cell types found in human peripheral blood. Purity of the populations was assessed by flow cytometry after staining for cell surface proteins defining: T lymphocytes, CD3; B lymphocytes, CD19 and; monocytes, CD33. Each population was stained in at least two experiments. In all cases 86-91% of cells stained positively for the expected surface marker, and less than 3% of cells stained positively for the other markers. The sole exception was one

T lymphocyte preparation that stained 86% positive for CD3 and 7% positive for the NK marker CD56. The purity of RBC and granulocyte preparations each contained less than 0.2% (2/1,000) contaminating cells as assessed by microscopy. Platelet preparations, also assessed by microscopy, had fewer than 3 contaminating cells per 10 high power fields of dense platelets. Thus each of the preparations were highly enriched for populations of the indicated cell types.

Each cell type was exposed to CsA concentration for 30 minutes at 37°C (Figure 29). Each cell population had a CsA-sensitive phosphatase activity, with IC_{50}s of 5-16 μg/L for all cell types except RBCs (IC_{50} = 220 μg/L, Table 10). The reason that the RBC dose response curve was shifted to the right is unknown. RBC have abundant CyP (233) and we hypothesized that membrane bound CyP may be sequestering CsA. Attempts at examining the effect of the RBC cell membrane by measuring CsA-sensitive activity in whole RBC versus RBC supernatants have met with mixed results.

These results suggest that all peripheral blood cell types behave very similarly with respect to CN inhibition by CsA, so that our methods of measuring clinical specimens properly reflected the effect of CsA on T lymphocytes. The only cell type not similar are RBCs, which are (almost) completely eliminated during PBMC preparation. The findings in RBC appear to account for the erratic and insensitive results obtained when attempts were made to measure CN activity in whole blood specimens from CsA-treated patients. These data also raised the question if CsA inhibited CN in other tissues as it did in circulating cells. If so, then CN inhibition may also account for the non-immune toxicities of CsA. To study this we turned to a mouse model to allow us to study both the *in vitro* and *in vivo* effects of CsA on the CN activity in non-immune solid tissues.

CN activity in murine cells and tissues

The effect of CsA on phosphatase activity was measured in T and B lymphocytes purified from mouse spleens (Figure 30). As shown, both populations exhibited CsA-sensitive phosphatase activity, with IC_{50}s of 38 μg/L (95% CI, 29-50 μg/L) for T lymphocytes and 23 (17-30 μg/L) for B lymphocytes. Populations of other circulating cell types could not be purified in sufficient quantity to include in these experiments.

Because both CyP and CN exhibit widespread distribution (see Chapter One), we tested the effect of CsA on whole organ homogenates of murine brain, heart, lung, spleen, kidney and thigh muscle (Figure 31). With the exception of brain, each of the tissues showed similar fractional inhibition of CN with increasing CsA concentrations. The IC_{50}s are reviewed in Table 10.

The lack of a CsA-sensitive (or FK-506-sensitive) phosphatase activity in brain was surprising considering the high levels of CN or CN mRNA found in brain extracts. To characterize the brain homogenates, experiments were performed mixing brain homogenates with those of either kidney or heart. Different proportions of each homogenate were used, as indicated in Figure 32, in the absence and presence of CsA 1000 μg/L. When equal volumes of brain and kidney homogenates were mixed, there was no CsA-sensitive activity, even though CsA-sensitive activity was demonstrated in the kidney homogenate (Figure 32A). Mixing brain with heart homogenates showed the expected degree of CsA-sensitivity when the ratio was 9 volumes heart: 1 volume brain, but somewhat less than expected CsA-sensitivity when equal volumes were used (Figure 32B). These data show that phosphatase activity in brain homogenates was not made CsA-sensitive by something present in homogenates from CsA-sensitive organs. Rather, the data suggest that there may be activity in the brain homogenates that makes the CsA-sensitivity in kidney, and perhaps heart, resistant to *in vitro* CsA.

The measurement of CsA blood and tissue levels in CsA-fed mice.

In order to test the effect of orally administered CsA on *in vivo* CN activity in murine organs, we undertook a series of experiments investigating the accumulation of CsA in blood and tissues in CsA-fed mice. In individual mice fed CsA by daily gavage, CsA blood and tissue levels were measured by HPLC 16 hours after the last dose. Figure 33 shows, for 13 experiments (3-7 mice per point), a linear relationship between trough CsA blood levels and oral dose over the range of 12.5-150 mg/kg/day. Linear regression analysis (goodness of fit, $r^2 = 0.9686$) predicted that doses of 5-18 mg/kg/d would achieve trough levels spanning the human therapeutic range (100-400 μg/L). Despite identical gavage conditions, blood levels varied between individual mice and between experiments with no consistent strain variations, and was similar to the inter-individual variation seen in people.

The accumulation of CsA in the different organs of mice fed various concentrations of CsA (0-100 mg/kg/day) was measured by HPLC of tissue homogenates prepared 16 hours after the fifth daily dose of CsA (Figure 34). In addition, three animals receiving the highest dose (100 mg/kg/day) received a sixth dose and were sacrificed three hours later, in an attempt to identify fluxes in CsA concentrations in blood and tissues following an oral dose. There was a linear correlation between blood level and tissue accumulation, with the exception of brain which did not accumulate significant amounts of CsA, even at high blood concentrations. The tissue levels in the organs removed three hours after the last CsA dose remained proportional to the CsA blood levels, indicating that equilibration of CsA between blood and tissue compartments occurred very quickly.

Finally, we measured the CN activity in the tissues of 15 mice fed CsA once daily for 5 days. There were three animals in each dosage group (0, 30, 60, 90 and 120 mg/kg/day). Because of the variability of absorption (see Figure 33), assaying each organ individually gave a broad range of CsA blood levels and allowed a detailed *in vivo* dose response curve to be generated. As expected brain CN was not affected and heart, lung, kidney, spleen and muscle showed a sigmoidal dose response curve for CN inhibition by CsA. A repeat experiment yielded similar results. A summary of the effects of both *in vitro* and *in vivo* CsA inhibition of CN in murine organs is shown in Table 10. It is interesting that, as with human PBMC, the IC_{50} for *in vivo* CsA is higher than *in vitro* for all organs. Although the difference did not appear as marked, it should be observed that the *in vitro* IC_{50} for whole spleen is 4-6 X higher than in isolated B and T lymphocytes, suggesting that some of the CsA binding sites present *in vivo* are also present in the clarified tissue homogenates.

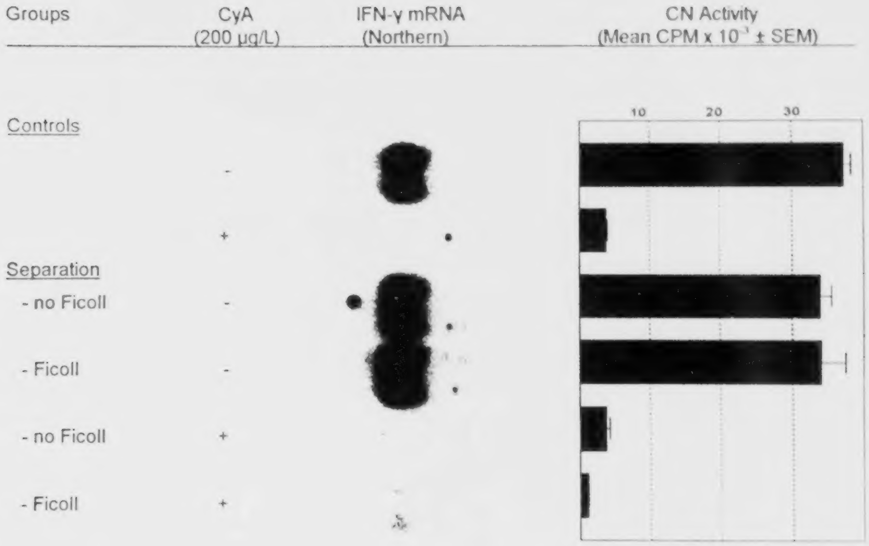


Figure 17: Effect of isolation procedure on calcineurin activity and cytokine induction. Control PBMC were exposed to CsA 200 µg/L *in vitro* and then assessed for calcineurin activity and cytokine gene induction before and after the same isolation sequence used for patient samples ("Ficoll"); a "no Ficoll" group was included as a control for separation time. CN activity shown as mean CPM ± SEM of triplicate samples.

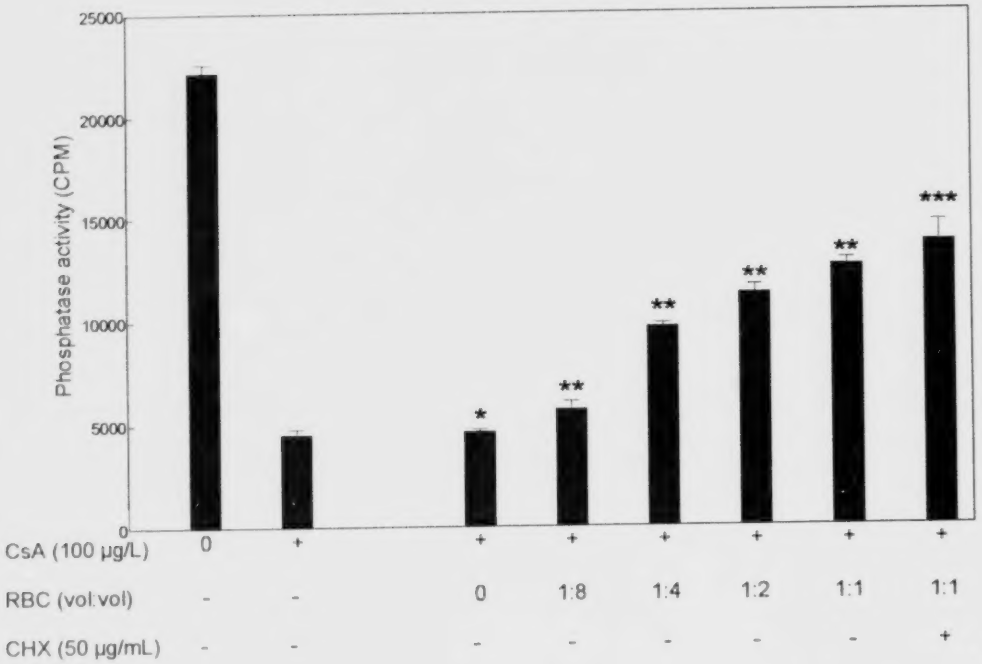


Figure 18: Effect of untreated RBC on the recovery of CN activity in CsA-treated PBMC. PBMC were treated as indicated with CsA *in vitro* for 1 hour, washed and resuspended at 10 X 10^6 cells/mL in medium and diluted 1:1(vol:vol) with packed RBC. RBC concentrations indicate dilutions starting with concentrated RBC. For example 1:1 indicates 1 volume PBMC (final concentration of 5 X 10^6 PBMC/mL) and 1 volume of undiluted RBC. After recovery with RBC, PBMC were reisolated by Ficoll-Hypaque centrifugation as before. Cycloheximide (CHX) was added, as indicated, prior to CsA treatment and maintained at 50µg/mL throughout the experiment. * p = N.S. compared to CsA-treated group before recovery; ** $p \leq 0.05$ compared to preceding group; *** p = N.S. compared to preceding group. Results represent mean ± STD of triplicate samples.

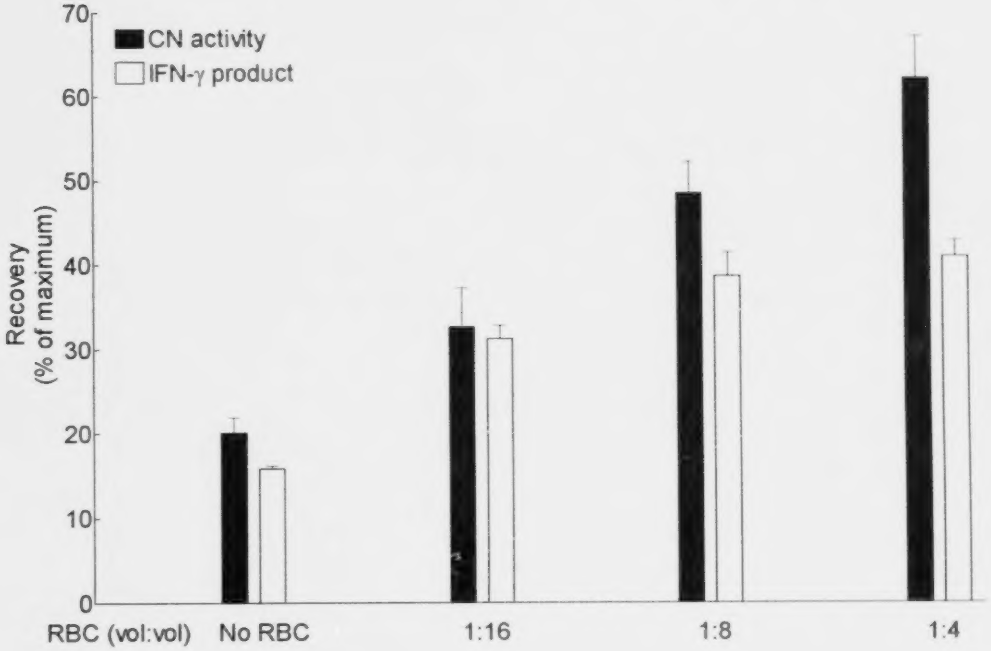


Figure 19: Recovery of CN activity and inducible IFN-γ secretion in CsA-treated PBMC. PBMC were treated with CsA 100 μg/L for 1 hour, washed and resuspended in medium + RBC, as indicated, for 4 hours. RBC dilutions represent the ratio (v/v) of packed RBC to PBMC-containing medium. Recovery represent mean ± STD of triplicate samples expressed as % of maximum possible recovery.

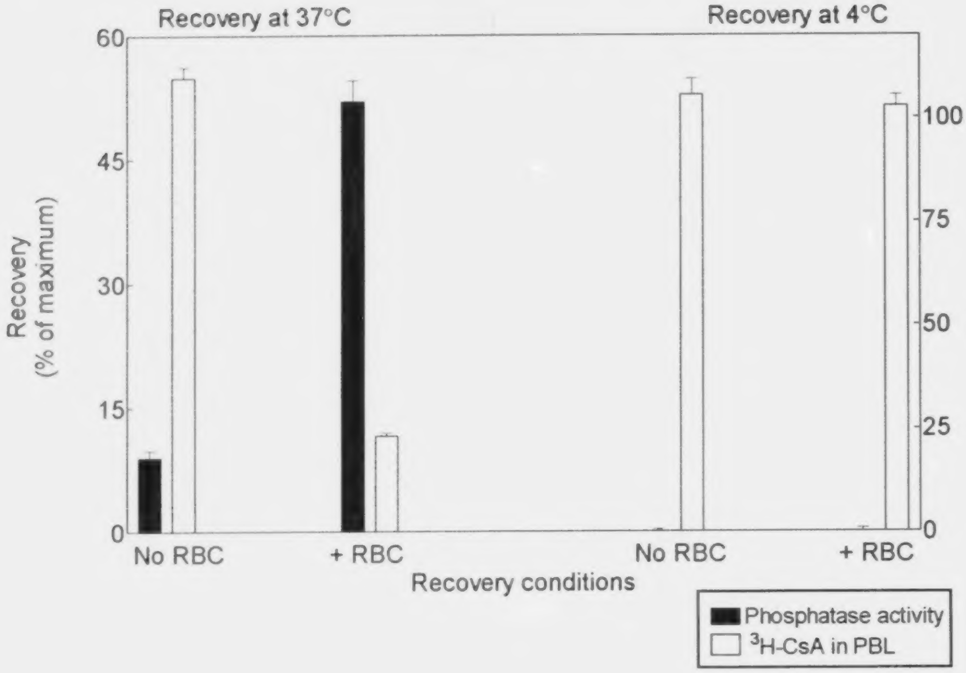


Figure 20: Effect of temperature on recovery of CN activity and on ^{3}H-CsA movement. PBMC were treated with ^{3}H-CsA 100µg/L for 1 hour, washed and resuspended in medium alone or medium with RBC (1:1 dilution) for 2 hours at 37°C or 4°C. Recovery shown as % of maximum possible recovery of CN activity; ^{3}H-CsA reflects dpm in 10^6 cells. All results represent mean ± STD of triplicate samples.

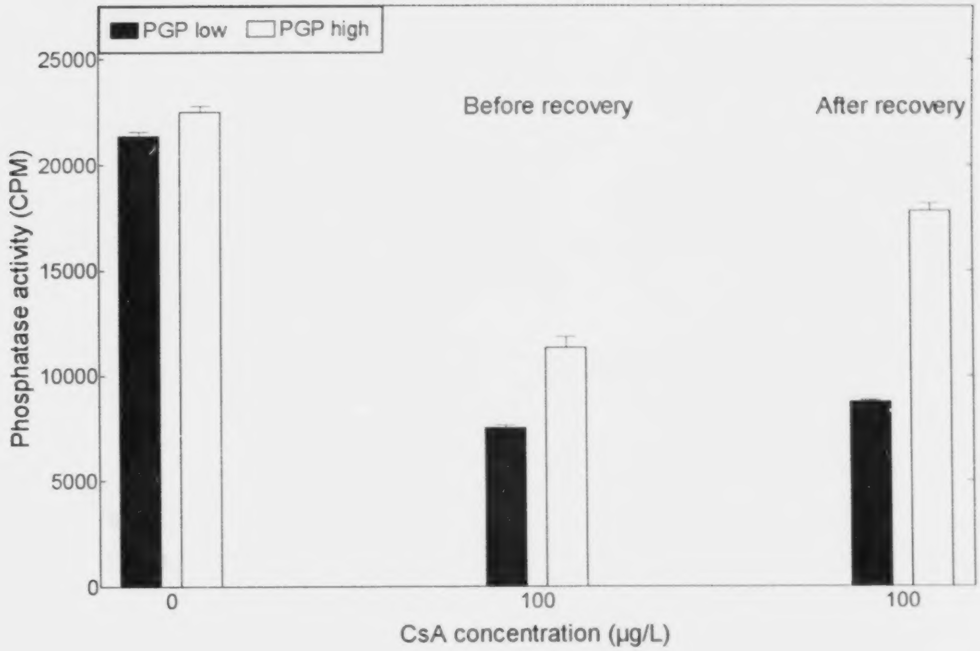


Figure 21: Effect of CsA on CN activity in cells with low or high expression of PGP. Cell lines differing only in surface PGP expression (see Materials and Methods) were assayed for phosphatase activity in the absence of CsA, immediately after exposure to CsA 100 µg/L for 1 hour ("Before recovery") and after being removed from CsA, washed and resuspended in CsA-free medium for 1 hour ("After recovery"). Bars represent mean ± STD of triplicate samples.

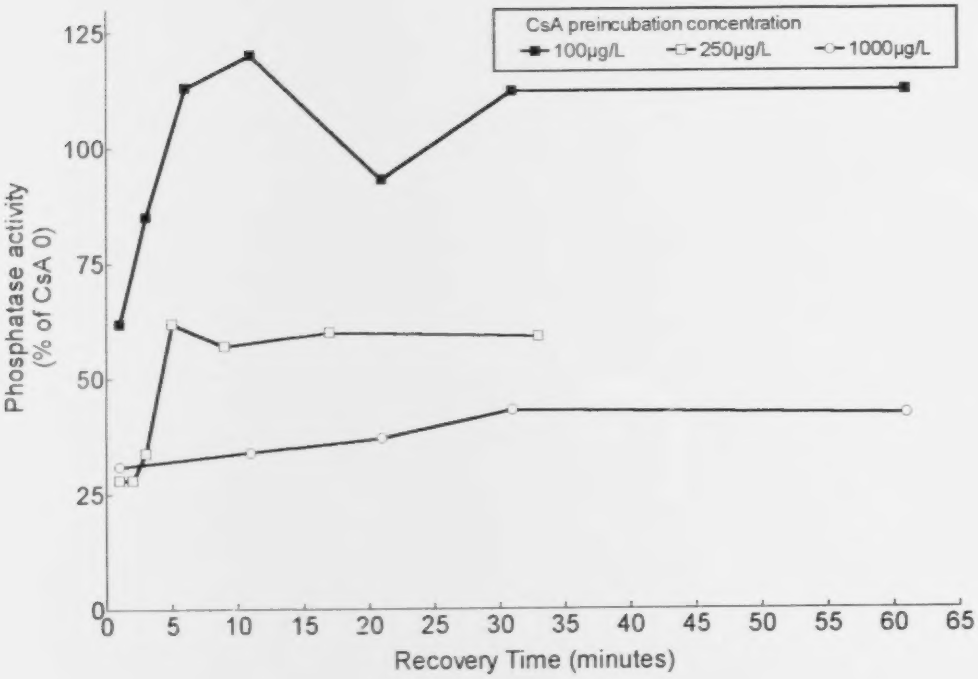


Figure 22: Time course of CN recovery in PGP-high cells. PGP-high cells (10 X 10^6/mL) were preincubated with CsA for 1 hour, washed in cold medium and brought up to 37°C with the addition of 9 volumes of warm medium. Cells were assayed for phosphatase activity at the times indicated. Each point represents the mean of triplicate samples.

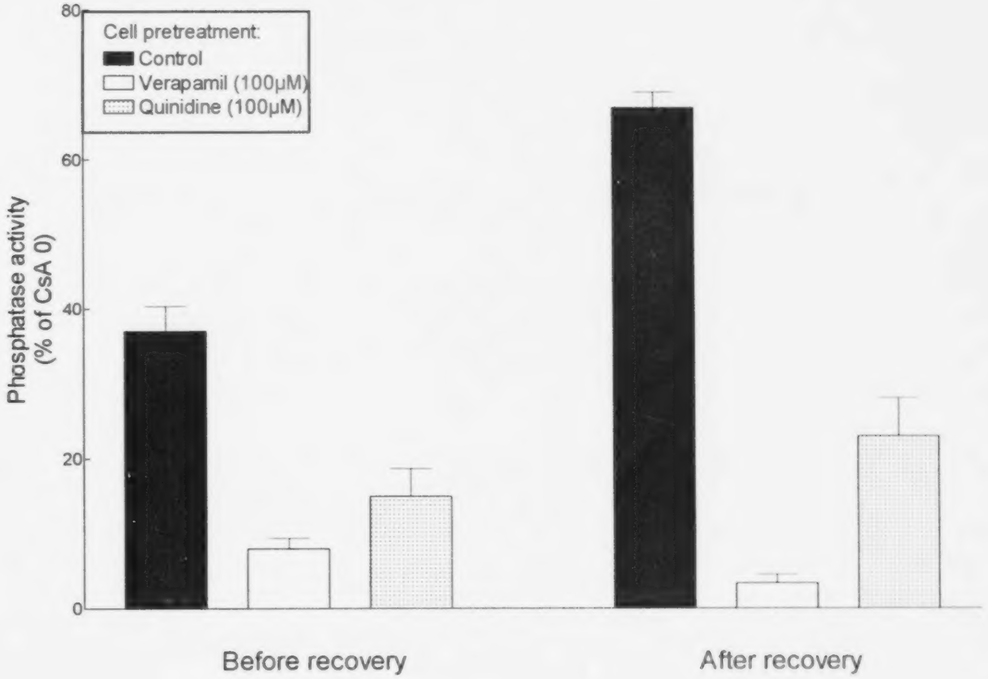


Figure 23: Effect of competitive PGP substrates on CsA transport by PGP in PGP-high cells. PGP-high cells were pretreated for 1 hour as indicated. CsA 100 µg/L was added and one hour later cells were lysed for measurement of phosphatase activity ("Before recovery"), or removed from CsA, washed and resuspension in CsA-free medium for 1 hour following which lysates were prepared and phosphatase activity measured ("After recovery"). Bars represent mean ± STD of triplicate samples.

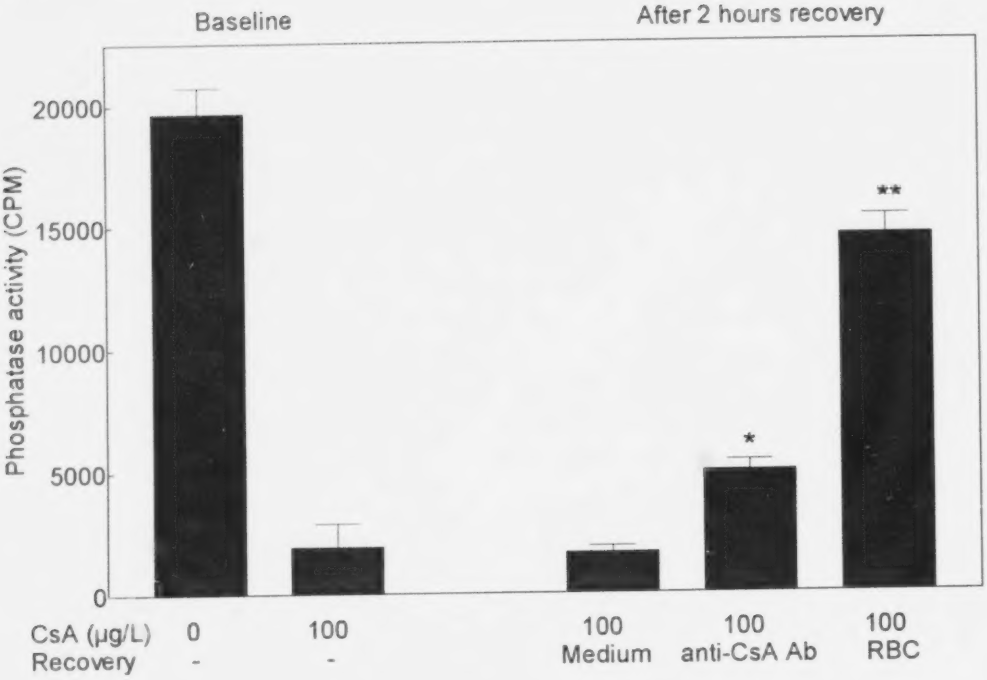


Figure 24: Effect of verapamil on anti-CsA Ab or RBC facilitated recovery of CN activity in PGP-high cells. PGP-high cells were exposed to verapamil 100 µM throughout the experiment starting 1 hour before exposure to CsA solvent ("CsA 0") or CsA 100 µg/L for 1 hour. Phosphatase activity was measured in the presence of CsA or solvent ("Baseline"). An aliquot of CsA-treated cells were washed and suspended in CsA-free medium alone, medium containing anti-CsA Ab (8.8µg/mL) or medium containing RBC (1:1 dilution). Two hours later phosphatase activity was measured. $^*p \leq 0.002$ compared to medium alone; $^{**}p \leq 0.0005$ compared to both medium alone and medium + anti-CsA Ab. Bars represent mean ± STD of triplicate samples.

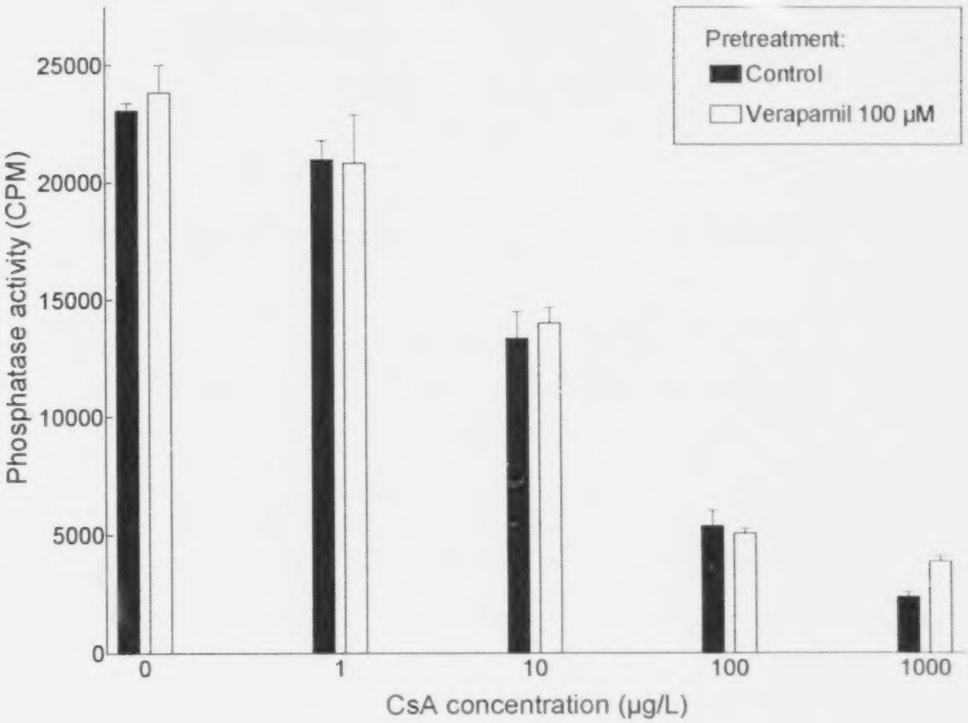


Figure 25: Effect of verapamil on CsA inhibition of CN activity in PBMC. PBMC (5 X 10^6) were exposed to either verapamil 100 µM or verapamil solvent (water) throughout the experiment starting 1 hour before exposure to CsA (concentrations as indicated). Phosphatase measurement was performed after 1 hour exposure to CsA. Bars represent mean ± STD of triplicate samples.

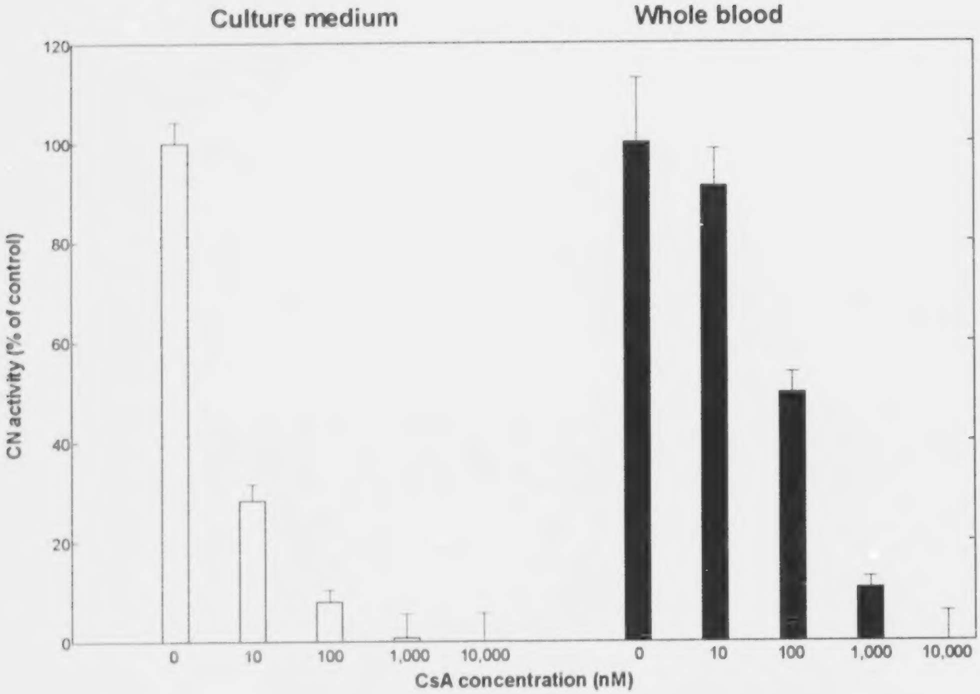


Figure 26: The dose response effect of CsA on CN activity in PBMC in culture medium versus whole blood. CN activity was measured in PBMC (5 X 10^6/sample) that were exposed to CsA either before isolation from whole blood or after isolation and resuspension in culture medium. Bars represent mean ± STD of triplicate samples.

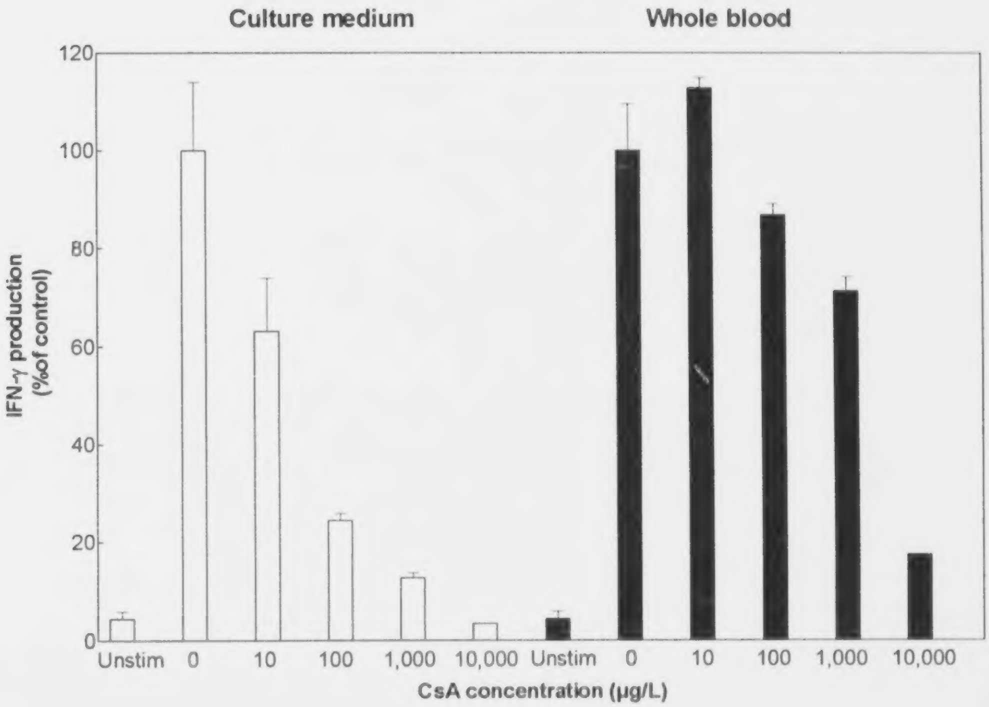


Figure 27: The dose response effect of CsA on inducible IFN-γ production by PBMC in culture medium versus whole blood. PBMC exposed to CsA in either culture medium or whole blood were washed, resuspended in CsA-free medium and stimulated by anti-CD3 (OKT3) 10 ng/mL for 18 hours. IFN-γ was then measured by ELISA in the cell supernatant. Bars represent mean ± STD of triplicate samples.

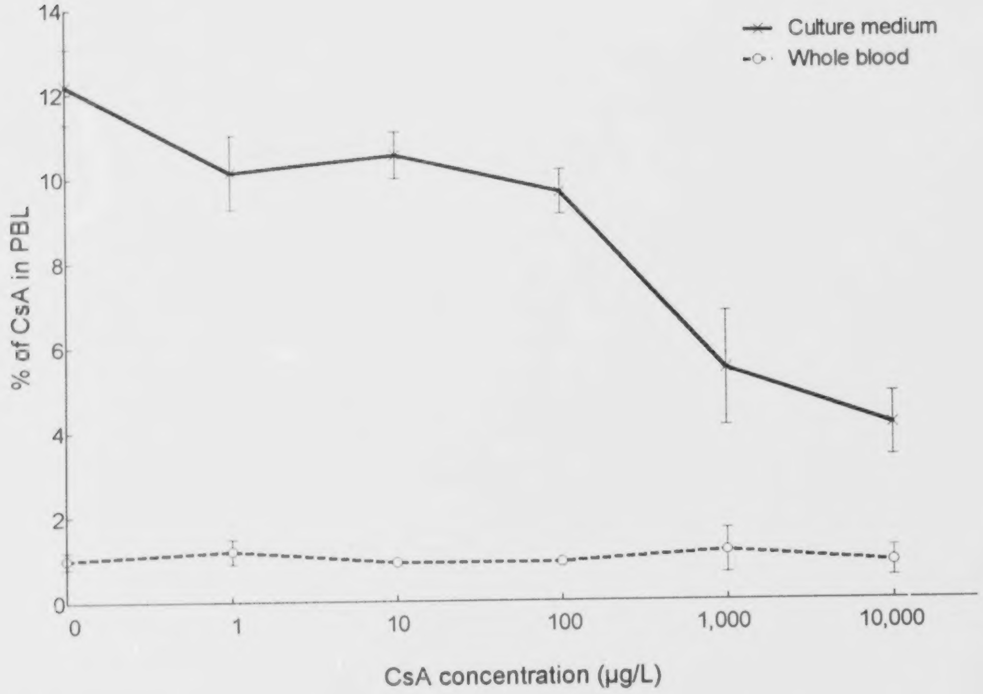


Figure 28: The accumulation of ^{3}H-CsA in PBMC in culture medium versus PBMC in whole blood. PBMC in either culture medium or whole blood were exposed to ^{3}H-CsA plus the indicated concentration of unlabelled CsA. The PBMC were isolated and the ^{3}H-CsA content was determined by scintillation counting. The amount of ^{3}H-CsA in PBMC is shown as the fraction of the total ^{3}H-CsA added. Results shown are composite means from 3 separate experiments ± SEM. Culture medium was significantly higher ($p \leq 0.02$) at all points.

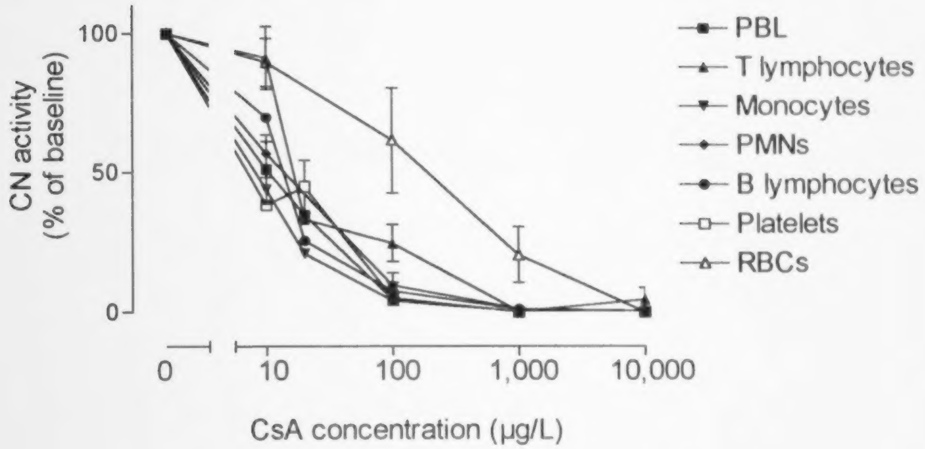


Figure 29: CN inhibition in human circulating cells treated with *in vitro* CsA. Populations enriched for the indicated cell types were purified (as described in Material and Methods), exposed to CsA for 30 minutes at 37°C, lysed and CN activity measured. Three to seven individual experiments were performed for each cell type and results normalized against CsA 0 group. Means from each experiment were pooled and are shown as mean ± SEM.

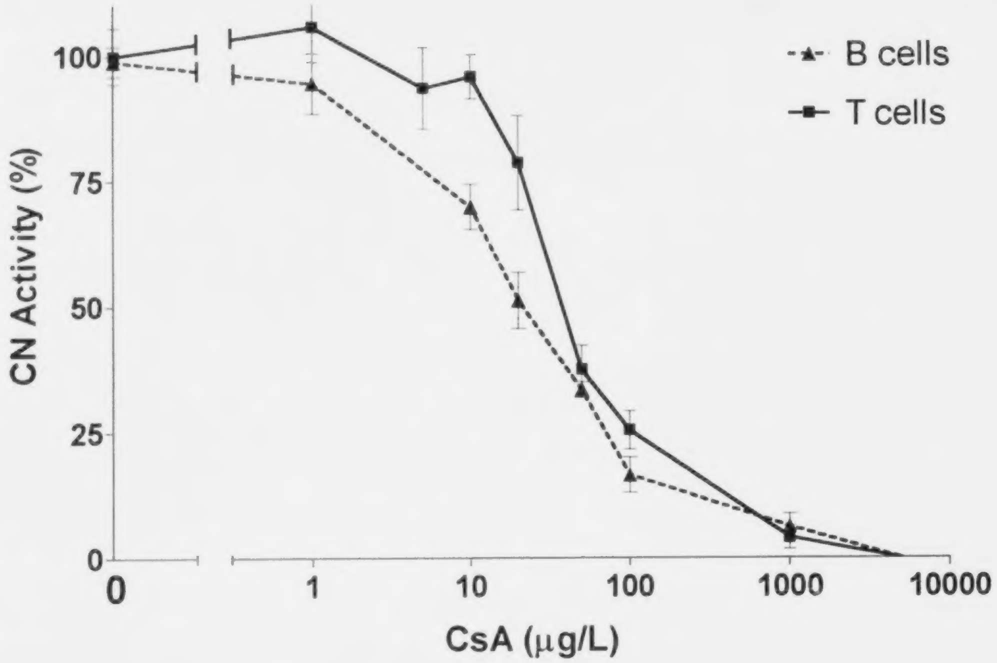


Figure 30: CN inhibition in murine splenic T and B lymphocytes treated with *in vitro* CsA. Populations of T and B cells were isolated using nylon wool. CN activity, in 3 X 10^6 cells/sample, was measured after *in vitro* exposure to CsA for 1 hour. The results show the mean ± STD of five individual experiments.

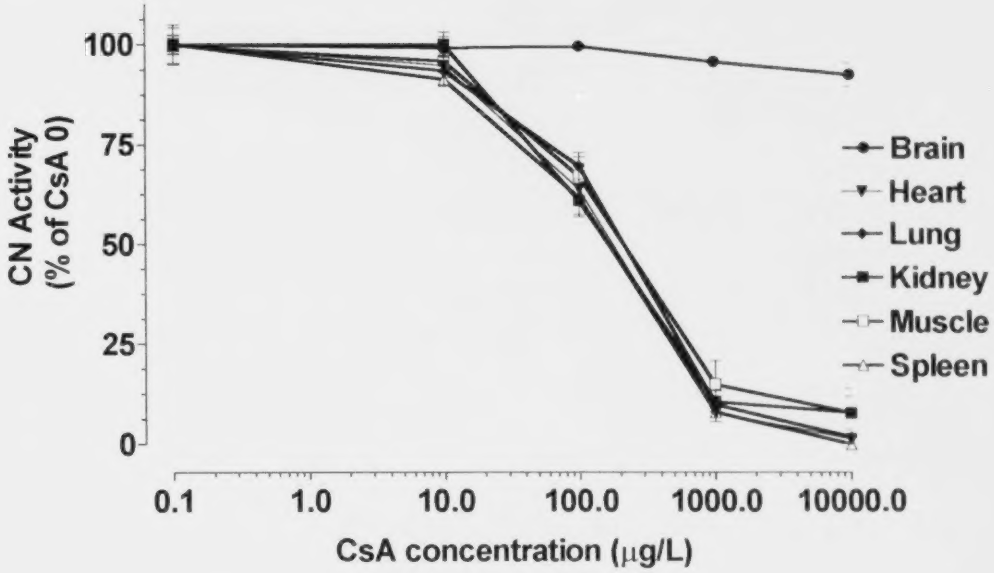


Figure 31: CN inhibition in homogenized murine tissues treated with *in vitro* CsA. Organs from BALB/c mice were harvested, frozen in liquid nitrogen, suspended in lysis buffer then homogenized with a Polytron. The lysates were centrifuged, the supernatants removed and centrifuged again. The clarified supernatants were then divided and exposed to CsA for 30 minutes at 37°C prior to measurement of CN activity. Results from four identical experiments were pooled and are shown as mean ± STD.

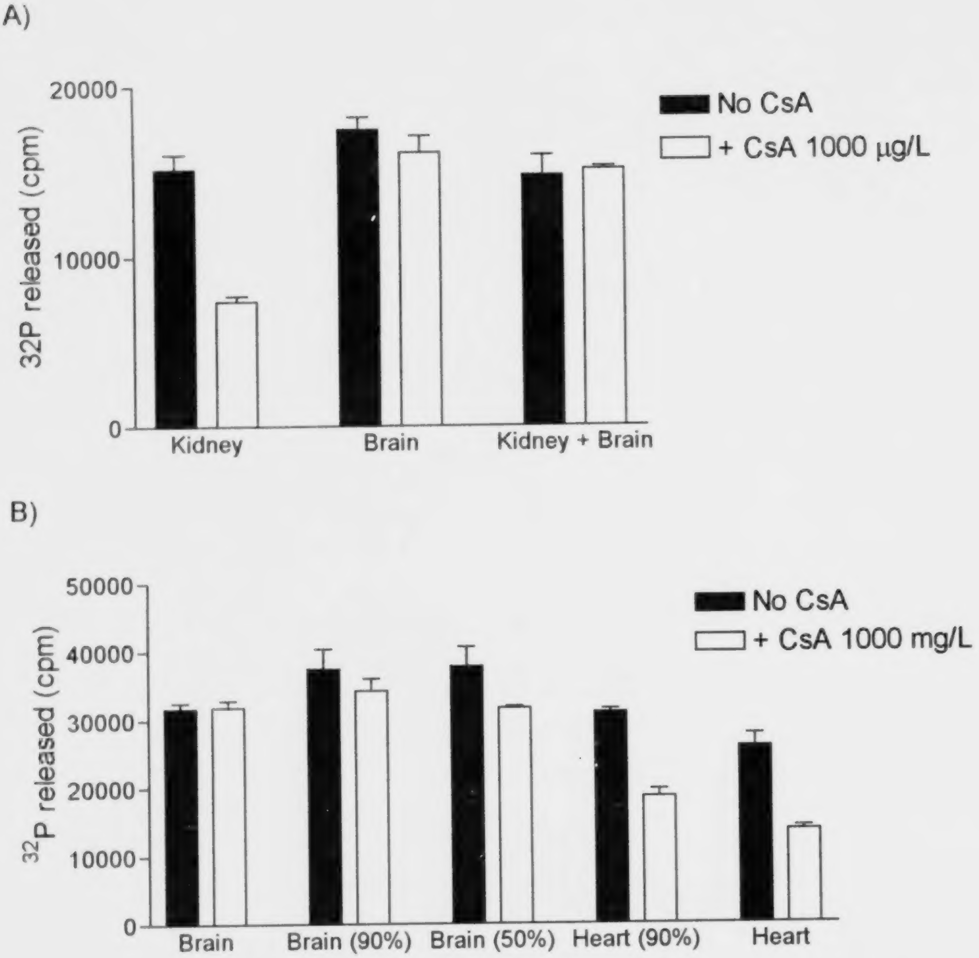


Figure 32: Phosphatase activity in mixtures of homogenates of murine organs. Murine brain, kidney and heart homogenates were prepared (Materials and Methods). A) Brain and kidney homogenates and a group containing equal volumes of both were incubated with CsA solvent or CsA 1000 µg/L for 30 minutes at 37°C prior to measurement of phosphatase activity. B) Brain and heart homogenates and groups containing brain:heart ratios of 9:1 (Brain 90%), 5:5 (Brain 50%) or 1:9 (Heart 90%) were treated as above. Phosphatase activity is reported as mean ± STD of triplicate samples.

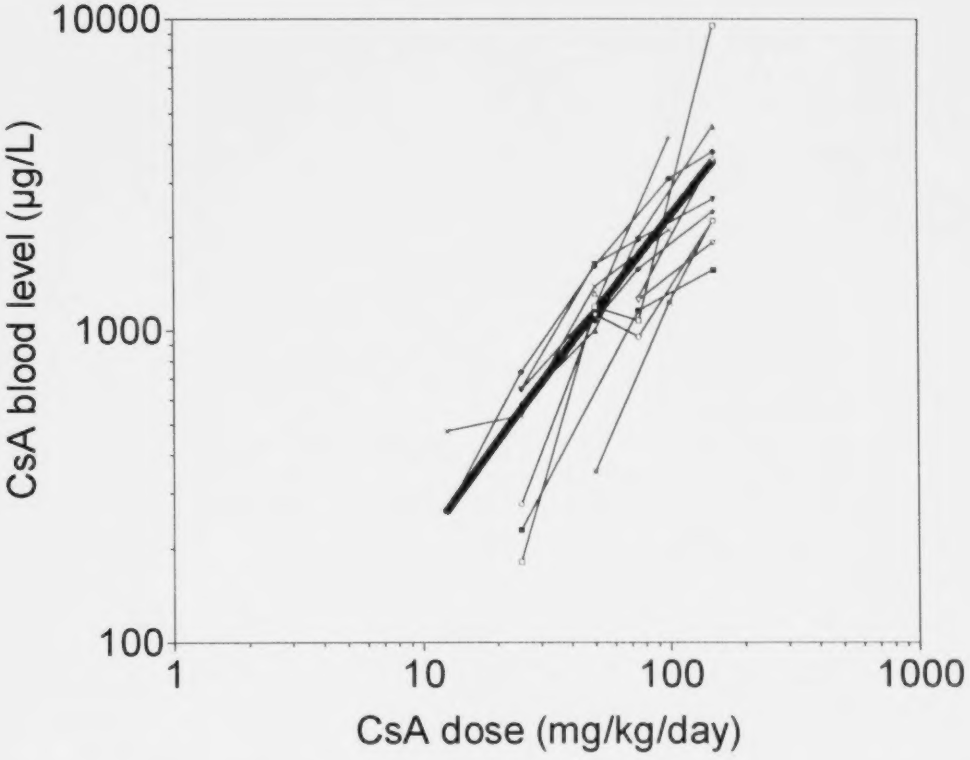


Figure 33: The relationship between the oral CsA dose and the whole blood CsA level measured by HPLC in 13 experiments. Mice fed CsA daily for 3-7 days were bled 16 hours after the last dose. The heavy line represents the linear regression for the mean of the experiments. The oral dose calculated to deliver the ranges corresponding to human therapeutic range of trough CsA concentrations was 5 mg/kg/d for 100 μg/L and 18 mg/kg/d for 400 μg/L.

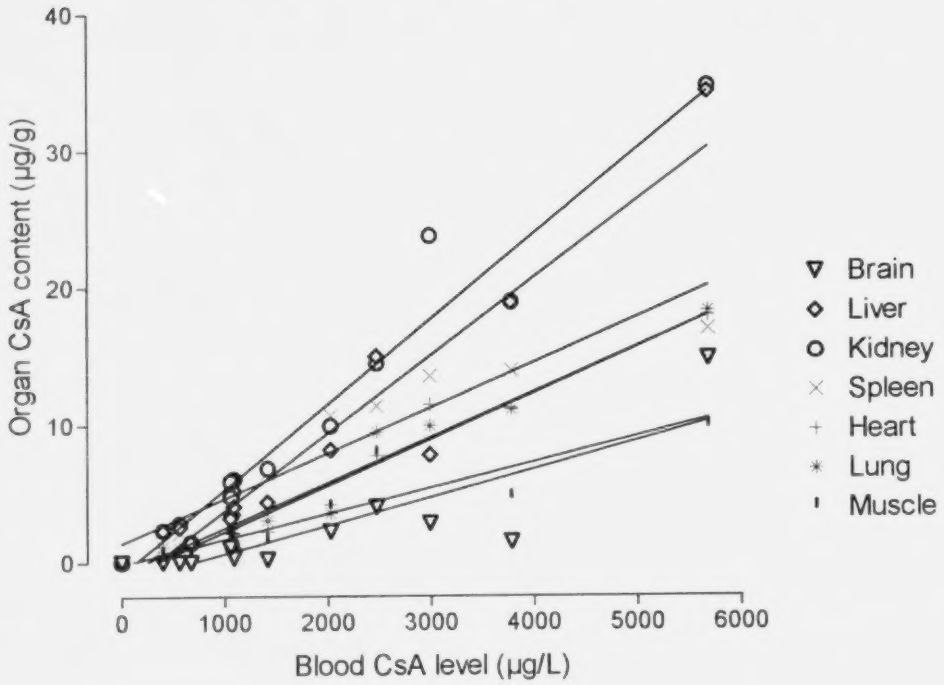


Figure 34: CsA accumulation in tissues of mice fed oral CsA for 5 days. Mice were fed CsA once daily at doses of 0-120 mg/kg/day. Sixteen hours after the last dose, organs were harvested, homogenized and CsA measured by HPLC. Three animals received a sixth dose of CsA and were sacrificed three hours later. Shown are individual data points and linear regression curve for each organ.

Table 7. Recovery of calcineurin activity after inhibition by CsA

Exp.	CsA (μg/L)*	% inhibition[‡]	Time (hours)[§]	% recovery[¶]
1	1	37	16	18
	10	88	16	0
	100	99	16	0
	1000	100	16	0
2	1	20	24	50
	10	23	24	0
	100	93	24	16
	1000	100	24	5
3	40	95	4	17
	200	100	4	12
4	100	94	2	1
5	100	88	4	20
6	100	92	4	9
7	100	92	4	0
8	500	100	3	19

* PBMC were isolated and used on the same day for each experiment. Following isolation, PBMC were incubated for 1 hour in medium with the CsA concentration indicated, washed and resuspended in CsA-free medium.

[‡] % inhibition = CN activity inhibition as a % of maximum inhibitable CN activity

[§] Time = number of hours cells were resuspended in CsA-free medium.

[¶] Recovery shown as degree of CN recovery as a % of total possible recovery.

Table 8. Recovery of IFN-γ production after inhibition by CsA

Experiment	CsA (μg/L)*	Time‡	Stimulus§	% recovery¶
1	200	5 h	A23187 0.5μM	4%
		8 h	A23187 0.5μM	0%
2	200	5 h	A23187 5 μM	8%
3	200	5 h	A23187 5 μM	30%
4	100	8 h	A23187 5 μM	0%
5	100	19 h	OKT3 10 μg/L	15%

* PBMC were incubated for 1 hour in medium with CsA concentration indicated, washed and resuspended in CsA-free medium

‡ Time = number of hours cells were resuspended in CsA-free medium

§ A23187 was added for final 4 hours of recovery period; OKT3 was added for the final 18 hours.

¶ Recovery shown as proportion of IFN-γ produced as a % of baseline control; CsA-treated cells made no IFN-γ when stimulated in presence of indicated concentration of CsA.

Table 9: The effect of verapamil on the degree of recovery of CN activity in PBMC.

Pretreatment	Degree of recovery (% of maximum possible)		
	Recovery conditions		
	Culture medium	Anti-CsA Ab (8.8 µg/mL)	RBC (1:1 dilution)
No verapamil	0 ± 1%	35 ± 2%	36 ± 4%
Verapamil 100 µM	0 ± 0%	26 ± 1%	43 ± 3%

* $p \leq 0.001$ compared to medium alone group within the same verapamil treatment group.

Table 10: IC_{50}s for inhibition of CN by CsA in human circulating cells and murine tissue homogenates.

	In vitro CsA exposure		*In vivo* CsA exposure	
	IC_{50} (μg/L)	95% CI (μg/L)	IC_{50} (μg/L)	95% CI (μg/L)
HUMAN CELLS				
T lymphocytes	16	7-35	ND*	
B lymphocytes	13	10-17	ND	
Monocytes	8	7-10	ND	
Granulocytes	13	10-16	ND	
Platelets	5	0-4793	ND	
RBCs	221	210-231	ND	
MURINE TISSUES				
Heart	156	44-546	293	59-1449
Lung	192	19-1856	1220	1033-1442
Kidney	118	74-187	316	6-17400
Spleen	158	15-1709	741	314-1750
Muscle	171	17-1673	761	23-25000
Brain	-*		-*	

* ND = not determined

** no effect of CsA was seen on the phosphatase activity of brain homogenates.

Chapter Five

Consequences of CN Inhibition

In Chapter Three, the conditions for measuring CN activity and the inhibition of CN activity in PBMC by CsA, both *in vitro* and in CsA-treated patients were established. Chapter Four provided data indicating that the discrepancy between the concentrations needed for *in vitro* CN inhibition and those needed for CN inhibition in patients was due to competition for CsA by non-PBMC binding sites in whole blood. Recovery from CsA was unlikely in PBMC manipulated *in vitro* because there were no binding sites for the CsA outside of the PBMC. Finally, the use of heterogeneous cell populations within each PBMC preparation would not be materially different than that for experiments using purified cell populations, since CsA causes the same fractional CN inhibition in each of the PBMC subpopulations. Indeed, in almost all tissues tested inhibition of CN occurs following exposure to CsA, both *in vitro* or following systemic administration to the living mouse.

This chapter will present results of experiments designed to identify the cellular and molecular bases for CsA immunosuppression as it is used clinically. The first section will describe the CN activity and cytokine induction and physiologic effect in CsA-fed mice rejecting an allogeneic tumor. The second section describes experiments in which PBMC were exposed to CsA *in vitro* at concentrations that produce clinical levels of CN inhibition. The consequences of this partial CN inhibition on the downstream signaling events involved in lymphocyte activation and proliferation were then studied.

I. Effect Of Orally Administered CsA On A Murine Response To An *In vivo* Allogeneic Stimulus

Effect of *in vitro* CsA on murine spleen cells.

The 50% inhibition points (IC_{50}) for CsA inhibition of CN activity and of IFN-γ induction were determined for normal mouse spleen cells exposed to CsA *in vitro* (Figure 35). CsA inhibited CN activity with the IC_{50} in four experiments ranging from 8 to 24 μg/L (mean = 17 ± 8 μg/L). IFN-γ production by normal mouse spleen cells was stimulated *in vitro* with calcium ionophore A23187 1 μM, with induction of mRNA within 1 hour and IFN-γ protein by 4 hours. As with CN activity CsA inhibited the production of IFN-γ with IC_{50}s of 1-8 μg/L (mean of four experiments = 6 ± 3 μg/L).

To assess whether the effects of CsA persist after washing, spleen cells were exposed to *in vitro* CsA 200 μg/L for 1 hour and washed. Cells preincubated in CsA and washed showed persistent complete inhibition of IFN-γ mRNA induction (by both PCR and Northern blotting) after 60 minutes exposure to A23187 1 μM and a maximum of only 10% recovery of IFN-γ production following 4 hours exposure. These data confirmed our findings in human leukocytes (Figure 17) and permitted us to perform *ex vivo* studies investigating the effects of *in vivo* CsA exposure.

The effect of oral CsA on CN activity *in vivo*

In both spleen cell suspensions and spleen homogenates taken from mice receiving oral CsA daily for 5 consecutive days, CN activity was inhibited but only at high blood levels (Table 11). Thus based even on whole blood CsA levels, the IC_{50} for CsA inhibition of CN activity was at least one order of magnitude higher than that seen in the *in vitro* experiments, consistent with the discrepancy seen between clinical specimens from transplant recipients and PBMC exposed to CsA *in vitro*.

The *ex vivo* responses of spleen cells from CsA-fed mice

Since the effect of CsA persisted after washing, we examined the degree of inhibition of *ex vivo* cytokine mRNA and product induction. Mice (five/group) were fed CsA for two days, rested for two days, then fed for three days. Sixteen hours after the last dose, spleen cells were harvested and pooled. To internally control for potential recovery from the effects of CsA, an aliquot of spleen cells from the Sham-treated group were exposed to CsA 50 μg/L *in vitro* for 15 minutes prior to stimulation. Splenocytes were stimulated with A23187 and/or PMA for 1 hour *in vitro* (Figure 36). mRNA was isolated, amplified by RT-PCR and probed for both IFN-γ mRNA and IL-2 mRNA (used as a separate measure of a CsA-sensitive cytokine). IFN-γ and IL-2 mRNA induction was partially inhibited in spleen cells from mice receiving CsA 75 mg/kg/d (blood level 1251 μg/L) but still appeared greater than the induction seen in the *in vitro* treated cells. Complete inhibition was seen in the mice receiving 150 mg/kg/d (blood level 1924 μg/L), indicating that this system is not CsA resistant. These mRNA results were confirmed in two other experiments. Likewise, after stimulation with A23187 and PMA for 4 hours, IFN-γ protein formation, measured at 20 hours, was completely inhibited only at high doses of CsA (blood level 2263 μg/L, Table 12).

The effect of oral CsA on an allogeneic response

We then tested the effect of CsA on cytokine induction during an *in vivo* allogeneic response. Work from this lab previously showed that rejection of intraperitoneal P815 mastocytoma tumor cells by BALB/c mice is characterized by increased steady state levels of IFN-γ mRNA (by RT-PCR), and IFN-γ-dependent induction of MHC product expression in kidney (234-236). In this series of experiments, there was the expected strong induction of both splenic IFN-γ mRNA (Figure 37) as well as MHC class I and II by the allogeneic tumor, as measured by radiolabeled antibody binding to tissue homogenates (RABA) (Figure 38). Near complete inhibition of IFN-γ production *in vivo* was only achieved in mice receiving CsA 75-150 mg/kg/d. CsA blood levels correlated with the extent of inhibition of MHC induction. In two experiments, the IC_{50}s for class I and class II were 780 μg/L and 517 μg/L, respectively, in the first experiment (n=7 animals/group, Figure 38), and 886 and 675 μg/L, respectively in the second experiment (8 animals/group).

II. The Consequences Of Partial CN Inhibition On Downstream Signaling Events Involved In Cytokine Production In Human PBMC.

Thus *in vivo* CsA inhibition of CN is similar to the degree of inhibition of IFN-γ induction in mice responding to an *in vivo* allogeneic stimulus. To more precisely study the quantitative relationship between CN activity and IFN-γ induction, and identify the intracellular signaling events affected, we turned to an *in vitro* model.

In Chapter Three, we showed that CsA-treated patients have approximately 50% CN inhibition in their PBMC for most of the day. To establish the proper CsA concentrations required to replicate this degree of inhibition in PBMC *in vitro*, we measured CN activity in PBMC exposed to CsA concentrations of 0 (solvent), 1, 2, 5, 10, 20, 50, 200 and 1000 μg/L (Figure 38). The results show the mean ± STD of four individual experiments. The mean IC_{50} was 10 μg/L (95% CI, 9-14 μg/L); individual IC_{50}s ranged from 8 μg/L to 23 μg/L. The data fit a sigmoidal dose response curve with a goodness of fit of $r^2 = 0.9995$.

Having established the appropriate range of *in vitro* CsA, we then investigated the steps involved in cytokine activation in T lymphocytes that occur subsequent to CN activation, including NFAT activation, DNA binding of nuclear proteins from stimulated cells, mRNA induction and cytokine secretion during short term stimulation. We followed this by examining the effect of clinical levels of CN inhibition on proliferation of PBMC responding during a MLR.

Activation of NFAT

The first step following CN activation is the dephosphorylation of NFAT, leading to a reduction in its apparent molecular weight (183,199). To examine this step in human cells, immunoblotting of extracts from freshly isolated T lymphocytes was performed (Figure 40) with a polyclonal anti-NFATp antibody (67.1). The cells had been exposed to CsA for 30 minutes prior to stimulation with A23187 5 μM for five minutes. Whole cell extracts were prepared, proteins separated by 6% SDS-PAGE, transferred to nitrocellulose and immunoblotted. Extracts from unstimulated cells (Lane 1) showed a sharp band at $\approx$ 140 kD. Stimulation in the absence of CsA results in the disappearance of this band and the appearance of a new band at $\approx$ 120 kD. These bands are consistent in size with phosphorylated and dephosphorylated forms of NFATp, respectively (183,199). The bands were quantitated using densitometry. With increasing concentrations of CsA, the ratio of the upper band (phosphorylated NFATp) to the sum of both bands increased, indicating that dephosphorylation was inhibited by CsA in a concentration dependent manner, with an IC_{50} of 20 µg/L (95% CI, 15-26). Similar results were seen on repeat Western blotting. Western blotting of PBL preparations not enriched for T lymphocytes were too insensitive to detect these changes. The IC_{50} was slightly higher than that for CsA inhibition of CN, but this antibody was raised in rabbits against murine epitopes. When similar experiments are performed in murine spleen cells, the IC_{50}s ranged between 7 μg/L for a crude splenocyte preparation and 18 μg/L for purified splenic T cells (Kung and Halloran, unpublished data).

DNA binding activity

We examined the effect of CsA on the DNA binding characteristics of nuclear proteins from stimulated PBMC. Nuclear proteins from treated PBMC were incubated with a ^{32}P-labelled DNA probe with a sequence corresponding to the NFAT-like "P2" binding site from the human IFN-γ gene promoter (192). This mixture was then electrophoresed through a 6% TBE gel, dried and autoradiographed (Figure 41). Nuclear extracts from unstimulated cells showed no bands (Lane 1), whereas stimulation led to two bands (Lane 2), both of which were reduced with increasing concentrations of CsA (Lanes 3-8). Band quantitation by Bio-Imager showed an IC_{50} of 8 μg/L (95% CI, 5-12 μg/L) for each band.

Characterisation of the proteins and binding specificity involved in these bands was undertaken using the proteins from the stimulated group (Figure 41, Lane 2). Incubation of the nuclear extracts with an antibodies to NFATc or NFATp specifically eliminated both bands (Figure 42, Lanes 2 and 3), with anti-NFATc treatment leading to the appearance of a new, supershifted band. Also, preincubation of the proteins with non-radioactive DNA probes corresponding to the IFN-γ P2 site or the homologous P2 site from the human IL-4 promoter abolished the bands, whereas a mutant P2 probe had no effect. Thus the DNA binding activity in stimulated PBMC that was inhibited by CsA is recognized by two different anti-NFAT antibodies, and specifically bound NFAT binding sites. The presence of two bands may have represented the binding of NFAT alone (lower band) or NFAT in conjunction with AP-1 proteins (upper band), as has been shown with other NFAT activities (45,183).

To investigate the functional activity of this NFAT-like activity, we transfected into PBMC blasts a plasmid encoding three P2 sites upstream of an SV-40 promoter driven chloramphenicol acetyltransferase (CAT) gene. The transfectants were pooled, then aliquoted, and exposed to CsA for 30 minutes prior to exposure to 5 μM A23187. After 48 hours, cells were harvested and CAT activity measured (Figure 43). A23187 exposure led to a 15 fold increase in CAT activity compared to transfected cells not exposed to A23187. CsA pretreatment led to a stepwise reduction in CAT activity, with an IC_{50} of 19 μg/L (95% CI, 4-92 μg/L).

mRNA levels

We studied the effect of *in vitro* CsA on IFN-γ and IL-2 mRNA levels in PBMC stimulated by 5 μM A23187. Northern blots of PBMC stimulated for one hour show induction of both IFN-γ and IL-2 mRNA levels that were reduced with increasing CsA concentrations (Figure 44). Quantitation by Bio-Imager and correction for β-actin levels reveals an IC_{50} of 5 μg/L (95% CI, 4-7 μg/L) for IFN-γ and 5 μg/L (95% CI, 4-7 μg/L) for IL-2. Similar Northern blots and identical IC_{50}s

IC_{50}s were seen when PBMC were stimulated for four hours, indicating that CsA inhibition of mRNA induction did not simply shift the time course of induction during the first four hours.

IFN-γ secretion

To examine the consequences of reduced IFN-γ mRNA accumulation, we used an ELISA kit to measure IFN-γ protein secreted by PBMC stimulated by 5 μM A23187 for 18 hours (Figure 45). In two experiments, stimulation resulted in a 10-100 fold induction in IFN-γ in the supernatants. CsA inhibited accumulation of IFN-γ over the same concentration range described above, with IC_{50}s of 7 μg/L (95% CI, 5-9 μg/L) in both experiments.

Mixed lymphocyte reaction

Finally, we investigated the effect of CsA on proliferative responses in PBMC through physiologic cell surface receptors. Responder PBMC were mixed with irradiated stimulator PBMC in a typical one-way mixed lymphocyte reaction (MLR) in the absence or presence of CsA. For measurement of proliferation, ^{3}H-thymidine was added to cultures on day 5 and cells were harvested on day 6. For CN and IFN-γ measurement, harvesting of cells and supernatants, respectively, was performed on day 6. Proliferation and IFN-γ secretion were both induced during the MLR and as shown in Figure 46, all parameters were inhibited by treatment by CsA. The IC_{50}s were: proliferation, 11 μg/L (95% CI, 0-

7500 μg/L); CN activity, 8 μg/L (95% CI, 1-60 μg/L); IFN-γ accumulation 11 μg/L (95% CI, 0-1073 μg/L). The values for CN activity and IFN-γ accumulation were the same as those obtained in short term experiments, indicating that there was no significant metabolism of CsA nor reduction in its effect over the 6 day experiment.

Thus CsA inhibition of CN activity resulted in a similar degree of inhibition of alloantigen stimulated IFN-γ production by murine splenocytes. The advantage of this system is that the antigenic stimulus occurs *in vivo*, and that the surrogate measure of IFN-γ secretion, MHC induction, is stable enough that it will not be affected by the procedures needed to isolate the tissues. Although these same advantages were not present in our studies of *in vitro* PBMC, CsA had a quantitatively similar effect on each of the molecular steps subsequent to CN activation (Table 13). This relationship persisted even when the lymphocytes were stimulated by antigen during a MLR, suggesting that the artificial circumstances used to measure the molecular steps in lymphocyte cytokine gene activation accurately mimicked the effect of stimulating the lymphocytes through wholly physiologic signaling systems.

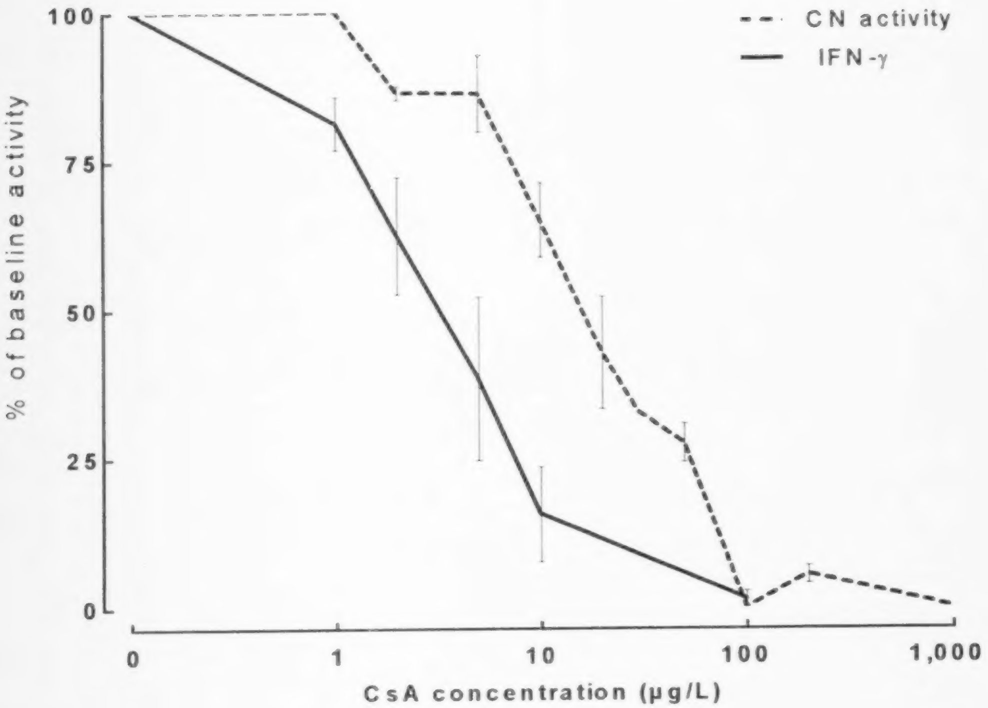


Figure 35: CsA *in vitro* inhibits CN activity and induction of IFN-γ production in murine spleen cells. BALB/c spleen cells (5 X 10^6) were incubated in CsA for 30 minutes at 37°. CN activity was then assayed as described. The mean IC_{50} is 17 ± 8 µg/L. For IFN-γ induction, CsA-treated spleen cells were stimulated with A23187 1 µM for 4 hours following which the supernatants were assayed for IFN-γ using an ELISA kit (Genzyme). The mean IC_{50} is 6 ± 3 µg/L. For both CN activity and IFN-γ production, results show the standardized mean ± SEM of four independent experiments.

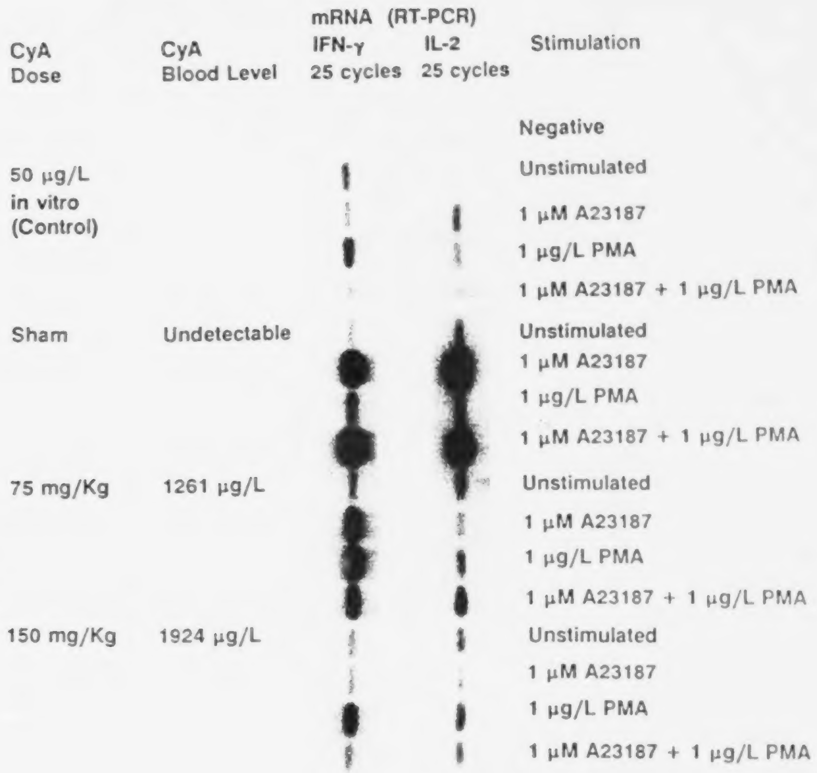


Figure 36: The effect of *in vivo* CsA treatment on the *ex vivo* induction of IFN-γ and IL-2 mRNA in BALB/c spleen cells in response to ionophore A23187 ± PMA. 5 X 10^6 spleen cells removed 16 hours after the last CsA dose from CsA-treated mice were stimulated as indicated for 60 minutes at 37°C. mRNA was extracted from the cells using cesium chloride and IFN-γ mRNA and IL-2 mRNA amplified by RT-PCR. The effect of *in vitro* exposure to CsA 50 μg/L in control splenocytes is included for comparison. The daily oral dose and the whole blood CsA level at the time of harvest for these groups of mice are shown.

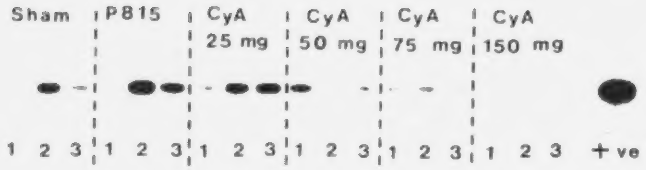
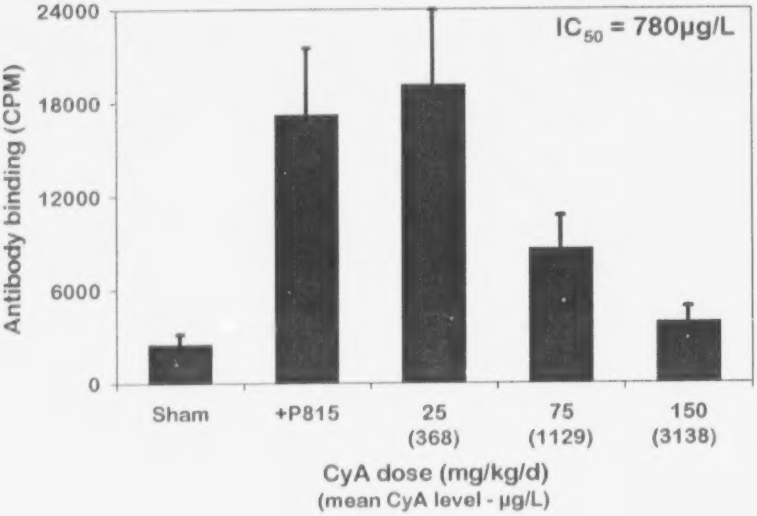


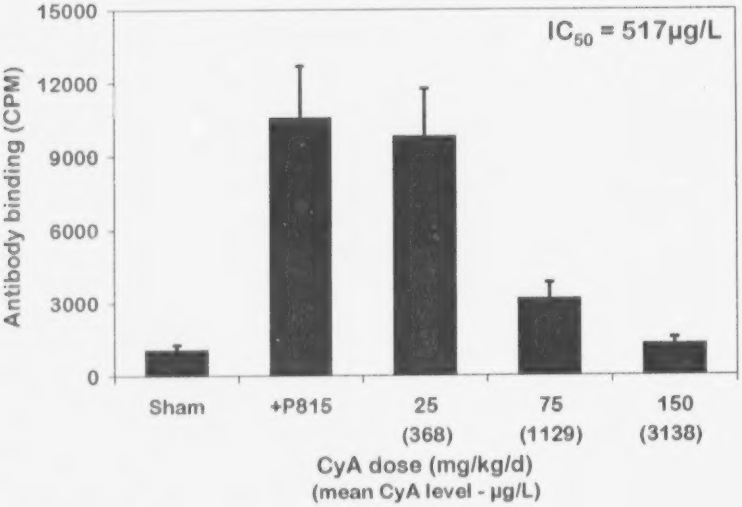
Figure 37: Oral CsA treatment inhibits induction of IFN-γ mRNA in spleens of mice rejecting P815 cells. CsA-treated mice received an intraperitoneal injection of 15-20 X10^6 P815 mastocytoma tumor cells. Seven days later, splenic mRNA was isolated, amplified by PCR using primers for murine IFN-γ, electrophoresed on an agarose gel, transferred to nitrocellulose, probed with a ^{32}P-labelled IFN-γ cDNA and autoradiographed. Each lane represents an individual mouse. Groups include control mice (Sham), mice receiving P815 but no CsA, and CsA-treated mice receiving P815.

Figure 38: The effect of CsA blood level on MHC product induction in kidney, assessed by binding of radiolabeled monoclonal Ab. BALB/c mice rejecting 15-20 X 10^6 allogeneic P815 tumor cells develop increased expression of MHC class I (A) and II (B) products in their kidneys which is inhibited by CsA. MHC expression was assessed by the binding of radiolabeled monoclonal anti-D^d or anti-I-A^d to homogenates of tissue (kidney) as described (25). The IC_{50}s for CsA inhibition of class I and class II expression were as indicated. Bars represent mean ± STD of seven animals in each group.

A)


IC$_{50}$ = 780µg/L
Antibody binding (CPM)
24000
18000
12000
6000
0
Sham
+P815
25
(368)
75
(1129)
150
(3138)
CyA dose (mg/kg/d)
(mean CyA level - µg/L)

B)


IC$_{50}$ = 517µg/L
Antibody binding (CPM)
15000
12000
9000
6000
3000
0
Sham
+P815
25
(368)
75
(1129)
150
(3138)
CyA dose (mg/kg/d)
(mean CyA level - µg/L)

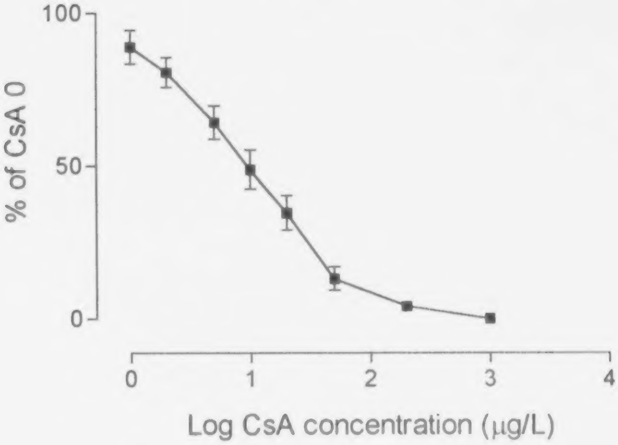


Figure 39: *In vitro* CsA inhibition of PBMC CN activity. PBMC were exposed to CsA at concentrations of 0 (solvent), 1, 2, 5, 10, 20, 50, 200 and 1000 μg/L. PBMC were lysed and CN activity measured. The results show the mean ± STD of four individual experiments. The mean IC_{50} was 10 μg/L (95% CI, 9-14 μg/L); individual IC_{50}s ranged from 8 μg/L to 23 μg/L.

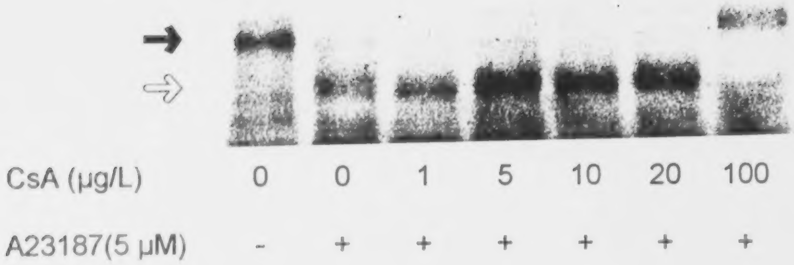


Figure 40: Anti-NFATp immunoblot of whole cell extracts from T lymphocytes stimulated for five minutes by A23187. T lymphocytes were purified from freshly isolated human PBL, exposed to CsA for 30 minutes prior to stimulation with A23187 5 μM (Lanes 2-7) for five minutes. Whole cell extracts were prepared, and for each group 200 μg protein separated by 6% (w/v) SDS-PAGE, transferred to nitrocellulose and immunoblotted with an anti-NFATp polyclonal antibody. Upper band (solid arrow) is at ≈ 140 kDa molecular weight and the lower band (white arrow) is at ≈ 120 kDa. Lane 1 - unstimulated PBL; Lane 2 CsA solvent control; Lane 3, CsA 1 μg/L; Lane 4, CsA 5 μg/L; Lane 5, CsA 10 μg/L; Lane 6, CsA 20 μg/L; Lane 7, CsA 100 μg/L.

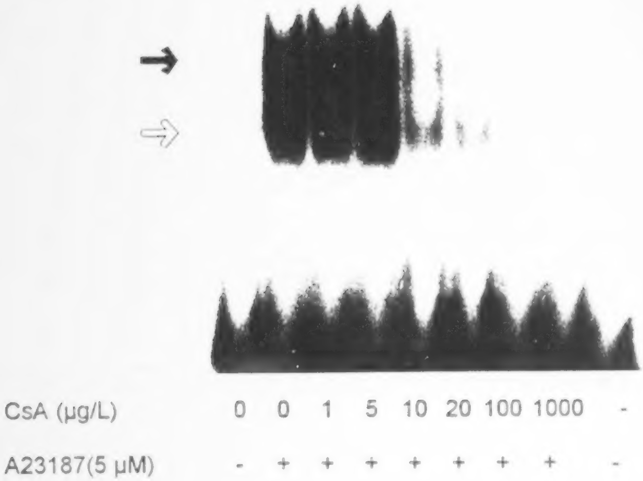

→
⇒
CsA (μg/L) 0 0 1 5 10 20 100 1000 -
A23187(5 μM) - + + + + + + + -

Figure 41: Effect of *in vitro* CsA on binding activity of nuclear proteins from stimulated PBMC to DNA. PBMC were treated with CsA, as indicated, for 30 minutes prior to stimulation with A23187 5 μM (Lanes 2-8) for five minutes. Nuclear proteins were isolated and then incubated with the ^{32}P-labelled DNA sequence corresponding to the "P2" NFAT binding site in the human IFN-γ promoter (192). The mixture were then run through a 6% (w/v) TBE gel, dried and autoradiographed. The lane on the far right is probe run through the gel in the absence of nuclear proteins. See the text for a description of the bands indicated by the arrows.

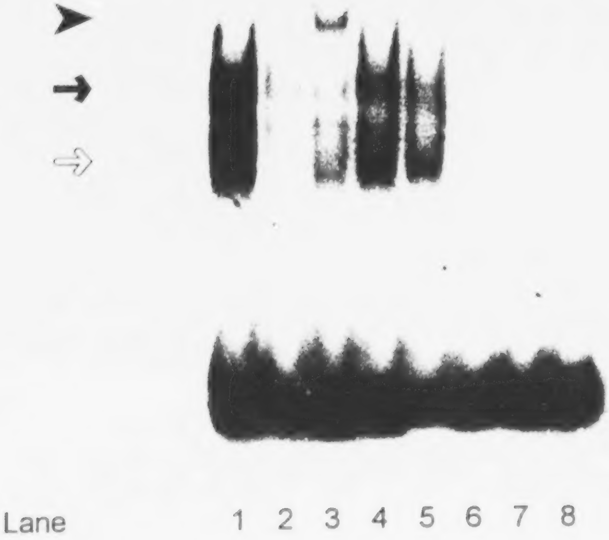


Figure 42: Characterisation of DNA-binding proteins from nuclei of stimulated PBMC. Nuclear extracts of stimulated PBMC (Lane 2, Figure 39) were incubated for 3 hours at room temperature with nothing (Lane 1), anti-NFATp polyclonal antibody (Lane 2), anti-NFATc monoclonal antibody (Lane 3), a non-specific control antibody (Lane 4), a mutant non radioactive P2 DNA sequence (Lane 5), non-radioactive P2 DNA (Lane 6) or a non-radioactive NFAT binding sequence from the IL-4 promoter (Lane 7). The mixtures were then incubated with the ^{32}P-labelled DNA probe described in Figure 41. The mixtures were then run through a 6% TBE gel, dried and autoradiographed. Lane 8 represents the DNA probe in the absence of nuclear proteins. See the text for a description of the bands indicated by the arrows.

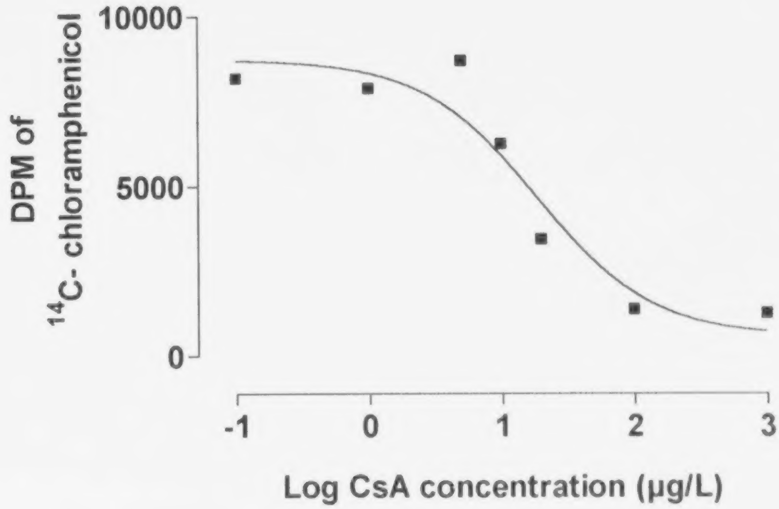


Figure 43: Effect of *in vitro* CsA on the induction of CAT activity in transfected PBMC. PBMC were exposed to 1% PHA for three days, washed and suspended in medium at 20 X 10^6/mL and incubated with 80 µL/mL of DNA encoding a plasmid containing three P2 sites upstream of an SV-40 promoter driven chloramphenicol acetyltransferase (CAT) gene. Electroporation was performed on aliquots of cells (see Materials and Methods). The transfectants were pooled, then split for 30 minutes CsA treatment prior to exposure to 5 µM A23187. After 48 hours, cells were harvested and CAT activity measured. Squares represent individual values, and the line represents the computer-generated sigmoidal-dose response curve generated from these values.

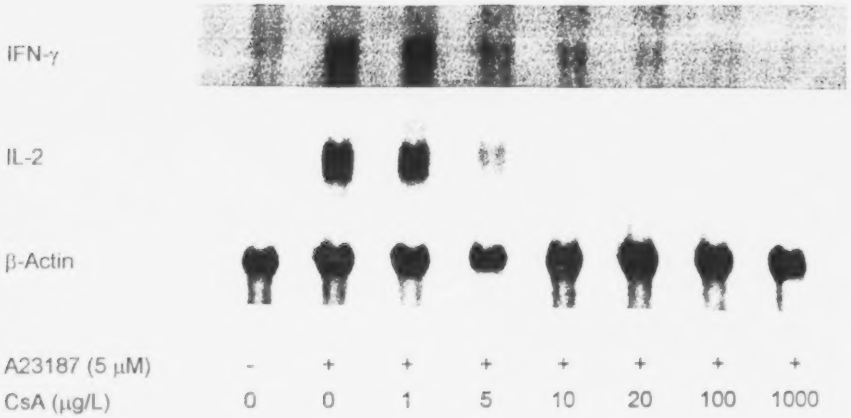

IFN-γ
IL-2
β-Actin
A23187 (5 μM) - + + + + + + +
CsA (μg/L) 0 0 1 5 10 20 100 1000

Figure 44: Effect of *in vitro* CsA on IFN-γ and IL-2 mRNA levels in stimulated PBMC. Following exposure to CsA, PBMC were stimulated with A23187 5 μM for one hour, RNA extracted with cesium chloride, run through an agarose gel, transferred to nitrocellulose and probed with ^{32}P-labelled cDNAs for human IFN-γ and human IL-2. The blots were the autoradiographed.

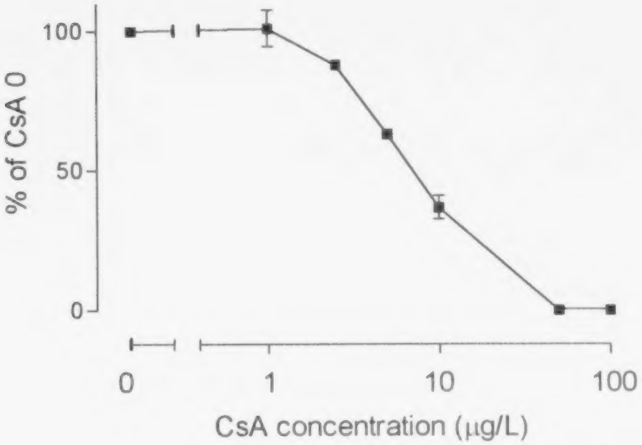


Figure 45: Effect of *in vitro* CsA on IFN-γ secretion by stimulated PBMC. PBMC were incubated with CsA for 30 minutes at 37°C followed by incubation with A23187 5 μM for 18 hours, at which time IFN-γ in the supernatants was measured by ELISA. The results represent the mean ± STD of two experiments normalized against the stimulated CsA 0 group.

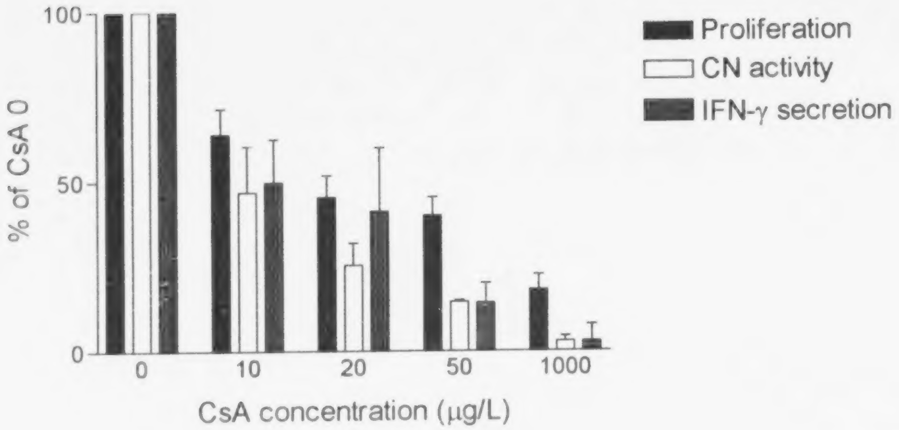


Figure 46: Effect of CsA on proliferation of PBMC, CN and IFN-γ accumulation after a six day MLR. PBMC were stimulated by irradiated stimulator PBMC. Six days later, proliferation was measured by measuring 3H-thymidine uptake. CN activity and IFN-γ were measured as previously described. Results represent standardized means ± STD of five experiments for proliferation and IFN-γ and three experiments for CN activity.

Table 11: CN inhibition in spleens of CsA-fed mice

Exp	Spleen prep	CsA Dose mg/kg/day	CsA Blood Level (μg/L)*	CN % control	IC_{50} (μg/L) mean (95%C.I.)
1	Cells	Sham	N.D.	(100)**	1170
	(n=5 mice/grp)	50	354 ± 36	104	(310-4420)
		100	1238 ± 62	46	
		150	2267 ± 331	18	
2	Cells	Sham	N.D.	(100)	< 1285
	(n=6-8 mice/grp)	75	1285 ± 111	33	
		150	3583 ± 568	21	
3	Homogenates	Sham	N.D.	(100)	< 856
	(n=3 mice/grp)	75	856 ± 47	27	
		150	2183 ± 85	11	

* whole blood TD_x, shown as mean ± SEM

** Sham fed (control) animals defined as 100%

Table 12. Effect of *in vivo* CsA treatment on IFN-γ production by spleen cells stimulated *ex vivo*

		IFN-γ Concentration in Supernatants (ng/mL)*	
CsA Dose	CsA Levels	Basal	After A23187 + PMA**
Sham-treated	undetected	43±1	6075±62
CsA 25 mg/kg/d	278±46 μg/L	42±1	3075±135
CsA 50 mg/kg/d	1133±578 μg/L	45±3	1500±54
CsA 75 mg/kg/d	960±261 μg/L	42±2	1500±182
CsA 150 mg/kg/d	2263±82 μg/L	45±3	175±6

* 10^6 BALB/c spleen cells were incubated in PMA (1 μg/L) + A23187 (1 μM), for 4 hours. IFN-γ was assessed by ELISA in supernatants at 20 hours.

** the estimated IC_{50} was 393 μg/L

Table 13: Summary of IC_{50}s of CsA effect on the calcium-dependent events involved in lymphocyte activation and proliferation.

	IC_{50} (μg/L) mean (95% CI)	Sigmoidal dose-response curve fit (r^2)
CN activity	10 (8-14)	0.9995
NFAT activation (Western blot)	20 (15-26)	0.9964
DNA binding (EMSA)	8 (5-12)	0.9958
Promoter activation (transfection)	19 (4-92)	0.9186
mRNA induction - IFN-γ	5 (4-7)	0.9974
- IL-2	5 (4-7)	0.9861
IFN-γ secretion	7 (5-9)	0.9995
Proliferation (6 day MLR)	11 (0-7500)	0.9977
CN activity (6 day MLR)	8 (1-60)	0.9993
IFN-γ secretion (6 day MLR)	11 (0-1073)	0.9975

Chapter Six

Discussion

I. Summary

The identification of CN as a direct molecular target of CsA gave us the opportunity to test the hypothesis that CN inhibition was the mechanism of action of this drug in patients. Early *in vitro* work suggested that CsA completely inhibited CN activity at concentrations below those achieved clinically, implying that this important calcium-activated pathway for lymphocyte activation was completely blocked in patients. In contrast, there was abundant clinical data demonstrating that lymphocyte activation was intact. Thus a paradox was evident and it was the intention of this project to resolve the differences between *in vitro* CsA effects on CN and the established clinical behavior of CsA-treated transplant recipients.

We first established that CN activity could be measured in freshly isolated human PBMC. Consistent with previous reports we found that *in vitro* CsA inhibited CN, with an IC_{50} of 10 μg/L and complete inhibition at concentrations above 100 μg/L. We then compared CN activity in various populations of CsA-treated transplant patients to CN activity in healthy controls. In all cases, patients had a 30-50% reduction in CN activity at trough CsA levels of 140-180 μg/L. When patients were studied over entire dosing intervals, we found that CN inhibition rose and fell in concert with CsA blood levels. Even at peak CsA concentrations that exceeded, on average, 800 μg/L in one group of patients, complete CN inhibition was uncommon and, when it occurred, very brief.

The difference between *in vitro* results and *in vivo* results was examined in detail. Recovery during specimen preparation was ruled out. Indeed, recovery in culture medium alone occurred very slowly and only to a limited extent. These findings helped us determine that the discrepancy between *in vitro* CsA effects and those found *in vivo* was due to the complex distribution of CsA within biologic fluids. The discrepancy could be reproduced *in vitro* by suspending PBMC in whole blood instead of culture medium prior to CsA treatment: elements in the whole blood limited the exposure of PBMC to CsA compared to the exposure seen at similar concentrations in culture medium.

We then determined if measurement of CN inhibition in heterogeneous PBMC mixtures reflected CsA effects on specific lymphocyte populations. First we showed that the production of lymphocyte specific cytokines by a heterogeneous PBMC population was inhibited by CsA to the same degree as CN. (Moreover, recovery of inducible IFN-γ production correlated with recovery of CN following CsA exposure.) Using purified populations of cells isolated from whole blood, we found that *in vitro* CsA inhibition occurred equally in almost all cell types, with the exception of RBCs. Moreover, CN activity in murine solid organs was inhibited by both *in vitro* CsA added to tissue homogenates, as well as *in vivo* CsA treatment of the mice. The only exception was in brain where CsA-sensitive phosphatase activity could not be demonstrated. Experiments to explain these latter findings are still needed.

Finally, having resolved the paradox of the *in vitro* versus the *in vivo* effects of CsA, we developed a model which we believe accounts for most of the immunosuppressive effects of CsA in transplant patients. First, we showed that CsA-treated mice with partial CN inhibition had a similar, partial inhibition of cytokine induction during an allogeneic immune response. This correlation between CN inhibition and inhibition of cytokine induction was examined in PBMC treated with CsA *in vitro*. Using the clinical findings of 50-70% CN inhibition, we found that this degree of CN inhibition resulted in a quantitatively identical reduction in each of the downstream steps involved in IFN-γ and IL-2 gene induction and protein secretion. Finally, the degree of CN inhibition was sustained during an MLR, and produced a quantitatively identical reduction in PBMC proliferation in response to allogeneic cells. Together these data support a model in which CN activation is a rate limiting step in T lymphocyte activation, and that partial inhibition by CsA leads to a partial inhibition of lymphocyte response. We have no evidence however that indicates whether CN inhibition is equal in all cells, or if there are populations of cells that are more sensitive to the CN inhibitory effects of CsA.

The implications of the clinical results, the distribution of CsA effect on CN and, finally, our model of the CsA effect in lymphocytes will each be discussed in detail.

II. CsA-Treated Patients Have Partial Inhibition Of CN Activity

The clinical results are exciting because they resolve the *in vitro/in vivo* paradox: CsA inhibits CN in PBMC both *in vitro* and *in vivo*, but its potency *in vitro* is considerably greater owing to a lack of other CsA-binding sites that are abundant *in vivo*. During the course of our work, two other groups have reported CN activity in CsA-treated transplant patients. Pai showed that bone marrow transplant recipients treated with CsA had a 73% reduction in CN activity compared to healthy controls and a 69% reduction compared to a similar cohort of patients not receiving CsA (237). Similarly, Piccinini showed that a group of CsA-treated adult renal transplant patients had a 40-70% reduction in CN activity compared to controls (238). Thus in several different groups of patients, we and others have shown that CN activity in PBMC of transplant patients receiving CsA is reduced by 40-70% compared to controls.

The partial inhibition of CN activity is consistent with a number of earlier reports that measured functional effects of CsA treatment. Yoshimura and Kahan reported a 40% reduction in IL-2 production by mitogen stimulated PBMC from CsA-treated patients (96), and in a similar population, Koutouby showed a similar reduction in cytokine mRNA levels using a quantitative PCR system (239). Finally, Cirillo studied the basophils of healthy volunteers who had received oral CsA for five days and showed a 40% reduction in histamine release compared to pre-CsA tests (240). Supporting studies in animals are difficult to find inasmuch as the relevant CsA doses and levels needed to reduce immunoreactivity to a vascularized allograft are undetermined. The data in this thesis present the first *in vivo* CN data in an animal model to date, albeit not in a vascularized graft model.

Recently, a mouse was reported in which the CN-Aα gene was knocked out by homologous recombination (the CN-KO mouse) (212). This mouse showed a 66% reduction in okadaic acid resistant, EGTA-sensitive phosphatase activity in T lymphocytes from spleen and lymph node. The remaining phosphatase activity was sensitive to *in vitro* CsA and FK-506. These animals showed evidence for both immunodeficiency and immunocompetance. The immune defect was manifest as reduced proliferation and cytokine production by T lymphocytes that were stimulated *in vitro* following an *in vivo* priming dose of hapten-protein. In contrast, T lymphocytes from CN-KO mice responded equally well as T lymphocytes from control animals following *in vitro* mitogen stimulation.

Thus CN-KO mice are similar to CsA-treated patients. The interpretation of differences between in immunocompetence in CN-KO mice and CsA-treated patients requires caution for two reasons. First, these are mice have developed in the absence of CN-Aα. Studies in which newborn mice have been fed CsA or FK-506 have shown defects in the phenotypic repertoire of circulating lymphocytes (241,242). The T and B lymphocyte repertoires were reported to be normal in the CN-KO mice. However genetic pressures at the time of embryogenesis may have encouraged other, subtle developmental defects that were not initially recognized. It should be noted, however, that children born of

CsA-treated mothers have had no consistently identified immunologic lesions (243,244). The second reason for caution is that, although the CsA-treated patients showed a similar degree of reduction in CN activity as the CN-KO mice, the precise CN holoenzyme(s) inhibited by CsA has/have not been identified. It is possible that CN-Aα is more or less important in human PBMC than it is in mice.

The measurement of CN activity may help overcome some of the pitfalls associated with measuring CsA drug levels. Many CsA assays detect not only parent CsA, but also CsA metabolites. The immunoassay used in the patients reported in Chapter 3 may overestimate parent CsA levels by 20-40% because of crossreactivity with CsA metabolites (245,246). Tissue levels of individual metabolites may exceed those of the parent CsA molecule, and these levels may fluctuate over time depending upon the condition of the patient (80,83). Moreover, the effect of individual CsA metabolites on CN activity is unknown. Thus the ability to measure the direct target of CsA immunosuppression would be appealing as it would obviate many of these other concerns. However at this point the inter- and intra-patient variability make this assay difficult to apply to an individual patient. Indeed, the only data correlating CN activity with a clinical event was the finding by Pai that among the CsA-treated bone marrow transplant recipients, the group that suffered GVHD had greater CN inhibition than the group that did not suffer GVHD (237). Because the immunologic mechanism(s) for GVHD is(are) not known, the value of CN monitoring in individual patients remains unestablished.

Even if not valuable for following individual patients, the measurement of CN inhibition may still be beneficial by allowing comparisons between groups receiving different pharmaceutical agents. The recent release of the CsA microemulsion (marketed as Neoral®) by Sandoz is such a circumstance. This formulation has vastly improved pharmacokinetics with respect to total amount of drug absorbed and time course of absorption compared to the standard CsA formulation (217,247,248). However, we found a slight but statistically insignificant increase in both peak CN inhibition as well as CN AUC suggesting that for the total population of patients, Neoral will have only a trivial effect on total immunosuppression. This is supported by early studies showing acute rejection in Neoral-treated patients is reduced by about 10% compared to the control group (249,250). While this subtle reduction in acute rejection rates may be due to a subtle increase in peak CN inhibition, it may also be due to the fact that periods of poor absorption are less likely to occur with Neoral. As a result, rejection episodes with the old formulation of CsA that may have been due to a relative lack of circulating CsA may not have occurred in the Neoral-treated patients.

Another comparison will be between currently available CsA formulations and the "generic" CsA formulations. The "generics" are rumored to be headed to market now that some of the original Sandoz patents for SandImmune® have expired. The data presented in this thesis indicate that CsA blood levels directly influence CN inhibition in PBMC. Just as SandImmune has been troubled by poor oral absorption, new CsA formulations will have to demonstrate adequate absorption. CN measurements may provide additional data in studies comparing these agents.

Finally, measurement of CN may make possible meaningful comparison of CsA with FK-506 in clinical trials. FK-506 has been licensed for use in liver transplantation in Canada and the U.S. but its role is currently unclear. The ability to compare CN inhibition in patients treated with these two agents would help to clarify the issues of immunosuppressive potency, as well as investigate reported differences in non-immune toxicities. Although differences between the agents have been reported, no clear way of comparing them has been available. Moreover, the "rescue" of some patients from intractable acute rejection by replacing their CsA with FK-506 has been reported (251). The investigation of such cases on the basis of direct measurement of the molecular target of these drugs would be of considerable interest.

III. CsA-Inhibition Of CN Activity Occurs In (Almost) All Cells And Tissues

Because isolation of sufficient numbers of T lymphocytes from patients would have required large volumes of blood, we worked with heterogeneous PBMC preparations. The studies designed to ensure that PBMC results accurately reflected the effect of CsA on T lymphocytes led to the interesting findings that CN inhibition by CsA takes place equally in almost all populations of cells tested. It should be noted that all studies performed reflect populations of cells; no assay were performed for individual cells. Studies of CsA effect on CN in homogenized murine organs further extended the findings that: a) CN inhibition occurs uniformly in most tissues, and; b) CN inhibition by *in vivo* CsA is less than that seen with *in vitro* CsA treatment.

The finding that CsA inhibition of CN requires lower *in vitro* concentrations was attributable, at least in part, to distribution of CsA to non-PBMC binding sites. This was shown by the findings that approximately 10 fold less ^{3}H-CsA accumulated in PBMC suspended in whole blood compared to those in culture medium. The distribution of CsA among the different components of blood has been extensively reported. Variations in hematocrit, lipoprotein and serum protein levels can each alter the distribution of CsA (80-82). Thus, since CsA binding sites are not saturated during maintenance immunosuppression, variations in blood composition and, perhaps, organ composition, could potentially have significant impact on CsA availability to PBMC, and thus inhibition of CN activity and cytokine synthesis.

Differences between *in vitro* and *in vivo* effects of CsA were not limited to circulating PBMC. Murine tissue homogenates also displayed lower IC_{50}s during *in vitro* treatment compared to *in vivo* CsA treatment. These findings were notable in light of the findings that all organs, except brain, accumulated more

CsA/g of tissue weight than was found in blood, suggesting that the true *in vivo* IC_{50}s may be even higher than we reported. These limitations notwithstanding, understanding the relationship between the dose response curve for different cells and tissues may be helpful. Even if the IC_{50} in the cell or organ of interest is slightly different than that in a more easily measured tissue, if the relationship stays the same, the measured compartment will be a useful surrogate for the relevant one. As an example, we have used measurement of CN inhibition of PBMC as a reflection of CsA effect on T lymphocytes.

The tissues that responded differently to CsA, with respect to CN activity, were human RBC and murine brain. The results in brain were not unprecedented. Studies of CN activity distribution in the rat also previously showed little response of brain homogenate serine phosphatase activity to CsA, despite abundant CN protein levels (140). One possible explanation is a relative lack of CyP. Although CyP is found ubiquitously, the brain has abundant CN and as such a low CyP:CN ratio mean that there are insufficient number of CsA:CyP complexes formed to measurably inhibit CN activity. Alternatively, not all CyPs mediate CsA inhibition of CN (252). Thus there may be abundant CyP expression, but if it is all a form that does not form a complex with CsA and CN, such as CyP-C, no CN inhibition would be expected. Both of these explanations would have to be applicable to the FK binding proteins as well, since brain homogenates are also resistant to FK-506. Two final possibilities include differences in CN holoenzyme. First, it is possible that the phosphatase activity we measured is CsA-resistant because it is not CN at all. The CN may be present in the particulate fraction of the homogenates that is removed during purification. That would indicate that CN in brain has different intracellular localization characteristics compared to CN found in the other five organs. Finally, it is possible that CN is present in the homogenate, but is resistant to CsA and FK-506 due to structural changes that prevent or minimize binding by the drug/immunophilin complexes. The fact that each of the three CN-A isozymes and most of the CN-B isoforms have variant forms due to alternative splicing mechanisms makes this explanation tenable. Much work is obviously required to solve this problem.

Compared to the absolute CsA-resistance seen in brain, the relative CsA-resistance seen in human RBC appears somewhat puzzling. RBC are sensitive to CsA, but require 10 fold higher concentrations than the other circulating cell types (RBC IC_{50} = 220 μg/L vs. 5-16 μg/L for the other cell types). As with brain, changes in the ratio of different CyP isoforms could shift the IC_{50} for CsA inhibition. Alternatively, RBC may possess a CN isoform that is relatively, but not absolutely, resistant to inhibition by CsA/CyP complexes. Finally, although the RBC results look very different when compared with other circulating cells, the CsA dose response curve for RBC is quite similar to that seen in the murine solid organs. Indeed, RBC differ from other circulating cells in that they do not respond to external stimuli by expressing new surface markers, secreting soluble mediators, or by proliferating and differentiating. Instead, RBC are like most solid organs that are designed to carry out a relatively limited array of specific functions. Thus it is possible that with the exception of RBC, circulating cells are inherently different in their biochemical makeup, and that these differences somehow make them more susceptible to CN inhibition by CsA.

Differences in susceptibility to CsA were seen in our studies involving human tumor cell lines that expressed different levels of surface PGP. Likewise, within patient populations the degree of CN inhibition correlated only weakly with CsA blood levels. It was common to observe within an experiment that different patients had widely varying CN activities despite similar, or even identical blood levels. These differences among people suggest varying degrees of susceptibility. While some of the variability may be due to technical errors associated with the preparation of PBMC from whole blood, this pattern was observed in dozens of experiments involving close to 200 patients. The explanations for differing susceptibility can be categorized as those affecting CsA entry into cells, those affecting CsA exit from cells and those that involve intracellular differences in the formation of CsA/CyP/CN complexes.

CsA is extremely hydrophobic and in our hands was able to enter PBMC and inhibit CN as quickly as we could measure. It is likely that CsA can enter cells merely by partitioning into the hydrophobic plasma membrane. Whether it can as easily pass out of cells is unknown. We identified at least two mechanisms by which CsA can exit. One involved the expression of high levels of surface PGP which allowed cells to quickly pump CsA out. This was very efficient, but saturable *in vitro*, although only when we used *in vitro* CsA concentrations > 100 μg/L. High PGP expression has been associated with tumor resistance to chemotherapeutic agents. Whether a similar mechanism could be responsible for "CsA resistance" in PBMC, either by induction of higher levels of PGP, or the selection of cells already expressing high levels of PGP is unknown. The non-PGP means of CsA efflux remains to be better characterized. Lastly, differences in the formation of CsA/CyP/CN complexes between individuals could account for differences in sensitivity to CsA. Altered kinetics for the formation of CsA/CyP/CN complexes may stem from structural differences in CyP, CN-A or CN-B, or by intracellular competition for CsA binding by other, currently uncharacterized sites.

Differences in individual sensitivity to CsA inhibition of CN in PBMC notwithstanding, the findings of widespread CN inhibition by CsA treatment provide some insights into the role(s) of CN in various tissues. In view of the large number of cells and tissues that exhibit CN inhibition by CsA, there are relatively few obvious functional or metabolic abnormalities. This would suggest that CN activity may not play a central role in the function of most cell types except, perhaps, lymphocytes. This idea is supported by the report that the CN-KO mouse did not manifest any obvious developmental abnormalities (212). In particular, neurologic abnormalities would be of interest due to findings that CN was important to the growth of chick dorsal root ganglia neurons (253) and the development of biochemical pathways associated with memory (229). Although we did not find any significant accumulation of CsA in the brains of CsA-fed mice, it is well recognized in the clinical literature that CsA can be associated with a variety of CNS lesions (75,254), most or all of which reverse upon discontinuation of CsA therapy. It is unknown whether these observations indicate that there are CNS cell types that are particularly susceptible to CsA, whether the blood-brain barrier has broken down in these patients allowing CsA into the brain, or if nervous system toxicities are indirectly mediated by CsA effects on other cells such as adjacent endothelial cells.

IV. CN Activation Is The Rate Limiting Step In Lymphocyte Activation

Previous studies identified CN activation as critical for the induction of cytokine gene transcription (126-128). However no reports have determined whether CN activation leads to an all-or-none activation of NFAT and the subsequent signaling events or whether NFAT activation and these events are quantitatively linked to CN activity. We became interested in this question because of our findings that CN activity is reduced but not eliminated in the lymphocytes of CsA-treated patients.

An advantage in using freshly isolated PBMC for these studies was that they produced cytokines following stimulation by calcium ionophores alone. The immunology literature is filled with unreferenced comments indicating that it is "widely known" that cells require two signals - a calcium ionophore and (usually) a phorbol ester (i.e., PMA). We have found this to be true of T lymphocyte cell lines, such as Jurkat, CEM and MOLT, but primary PBMC preparations do not require an additional exogenous signal. Our studies show that the addition of PMA shifts the CsA dose response curve to the right - i.e. makes cell activation less sensitive to CsA, perhaps due to activation of pathways that bypass CN. While it is possible that the responding cells in our PBMC preparations were receiving a signal in addition to the exogenous calcium ionophore, the MLR results indicate that such a signal does not substantially change the sensitivity to CsA. Together, these results suggest that PBMC have a lower activation threshold than many tumor cell lines. Whether this is true for all PBMC or a particular subset of cells is not known.

The findings that each of the events subsequent to CN activation is quantitatively linked to CN activity were novel. Our experiments studied the signal transduction pathway that involves NFAT activation. The reduction in NFAT-like DNA binding activity that occurred with reduced CN activity suggested that ongoing NFAT activation required ongoing CN activity, since even over a period of two days (transfection) or six days (MLR), the reduction in CN activity was not overcome. This indicated that NFAT dephosphorylation by CN was balanced by the inactivation of NFAT, either by nuclear export, rephosphorylation or both. The nature of this inactivating mechanism is unknown at present.

Shortly after the completion of our studies showing that CN inhibition led to a similar degree of inhibition of IFN-γ and IL-2 mRNA induction and IFN-γ (and probably IL-2, as indirectly measured by proliferation during the MLR) secretion, two groups reported the phenotypes of NFAT1 (formerly called NFATp) knock out mice (255,256). In both cases, the mice exhibited aberrent regulation of cytokines and immune responses, but both IL-2 and IFN-γ production were reported to be normal. This latter fact is somewhat surprising because while we have not characterized the protein involved in our NFAT-like activity, NFAT1 is the most abundant of the family members described and we would have predicted that IL-2 and IFN-γ would have been severely affected. Whether or not the "normal" IL-2 and IFN-γ levels ultimately prove to be truly normal, these mice have already added an element of complexity to the model of how CN and NFAT behave *in vivo*.

In addition to activation of NFAT family members, CN has been shown to play a role in the activation of other transcription factors. These include NF- κB, which is activated by the phosphorylation and degradation of its cytoplasmic inhibitor protein IκB (257) and Jun, which is activated by phosphorylation by Jun N-terminal kinase (JNK) (51). It is possible that a reduction in CN activity leads to a reduction in the activation of these pathways as well. However the *in vitro* proliferation data and the *in vivo* cytokine induction data suggest that the pathways do not work in an additive or a synergistic manner.

Is *in vitro* stimulation of PBMC with a calcium ionophore relevant? While the data were all consistent with a direct quantitative link between CN activity and downstream signal transduction events, the experiments were performed with a single concentration of a non-physiologic stimulus. While the MLR proliferation data and the *in vivo* murine data support this model, a broader view of stimulus strength is warranted. Clinically, differences in antigen strength are apparent in transplant patients. One example involves the findings that not all MHC antigen mismatches are equal. Indeed, MHC matching for individual donor/recipient pairs is poorly predictive of the likelihood of acute rejection. Also, while CsA has lowered the incidence of acute rejection in transplant recipients, there has been no significant increase in rates of infection. These results suggest that CsA treatment (and hence partial CN inhibition) may change the threshold for stimulation of lymphocyte activation. We have studied this to some extent and found that in populations of PBMC, partial CN inhibition led to relatively greater inhibition of weak activating stimuli (low concentrations of calcium ionophore or anti-CD3 monoclonal antibody) compared to strong stimuli (high concentrations) (230). However these studies were performed only *in vitro* and using only exogenous, and possibly nonphysiologic, stimuli. Moreover, single cell studies have not been performed to determine in partial inhibition of a CsA-sensitive step in a population of cells represents complete inhibition of some cells but no inhibition of others, or partial inhibition of all cells. Further study with a relevant model is needed and the CN-KO mouse may offer some advantages in such a model.

Is partial inhibition of lymphocyte activation the sole mechanism of CsA immunosuppression in vascularized organ transplantation? While it is tempting to ascribe all the clinical effects of CsA to a single mechanism, other possibilities must be entertained. Another hypothesis regarding the mechanism of CsA effect is that CsA induces the production of transforming growth factor β (TGF-β) by lymphocytes and renal tubular cells (258-260). These effects have only been demonstrated at *in vitro* concentrations of CsA >100 μg/L, which would completely inhibit CN, suggesting that CsA induction of TGF-β would occur indirectly. Moreover, since complete CN inhibition occurs rarely *in vivo*, this model needs to be shown to be clinically relevant.

Most effects of CsA are almost certainly due to inhibition of CN. Data supporting this hypothesis were presented in the introduction, and include the findings that CyP is the dominant intracellular CsA binding protein, that CyP is necessary but not sufficient for CsA-mediated immunosuppression and that immunosuppression by CsA and related homologues is tightly linked to CN inhibition. Effects of CsA not related to CN inhibition will require the identification of other binding molecules and their characterization as important in a different signaling or metabolic pathway(s).

While non-CN mechanisms of CsA effect remain hypothetical, the widespread inhibition of CN in a variety of cells presents the discreet possibility that CsA immunosuppression may be result of CN inhibition in other cells or cell pathways. While we have shown that CN inhibition prevents NFAT activation in lymphocytes, NFAT is found in most or all types of circulating leukocytes (196) as well as endothelial cells (EC) (188). A recent report showed that stimulation of GM-CSF production by EC was inhibited by CsA (261). While the specific role of GM-CSF production by EC is uncertain, this data suggests that EC activation may utilize a CN-dependent signaling pathway, and that other activation-dependent genes may be similarly controlled. The importance of EC in inflammation and transplant rejection is becoming increasingly appreciated. EC produce chemotactic factors that can recruit inflammatory cells, following which interactions can take place through adhesion molecules, which are expressed in response to cytokines and injury (262-264). Inhibition of any or all of these by CsA may be immunosuppressive by preventing the recruitment and stimulation of potentially reactive cells.

Finally, CN inhibition has been correlated with events not involved in gene transcription. Specifically, *in vitro* CsA inhibits the stimulation-dependent degranulation of cytotoxic T cells (265) and basophils (240). As discussed earlier, this latter study included experiments showing that histamine release from basophils was reduced by 40% in CsA-treated volunteers, indicating that this mechanism can operate *in vivo* at clinically relevant CsA concentrations. Thus, in addition to partially inhibiting the activation of naive lymphocytes, CsA may also interfere with some degranulation events in effector cells.

V. Future Directions

I believe the data presented here introduce a new paradigm for how CsA, and perhaps other immunosuppressants, should be studied. Since the early years of transplantation investigators have been designing *in vitro* and *in vivo* experiments to study the effects of immunosuppressive agents without having the faintest idea of how the drugs worked in their clinical target populations. Indeed, there is no monitoring system for corticosteroids which are used clinically in a dose range that spans three orders of magnitude. In contrast, CsA blood concentration monitoring may have been more harmful than helpful for basic science investigators since it is now apparent that for the last twenty years, *in vitro* studies have been using 10-100 times too much CsA. By showing the clinical effect of CsA on its target molecule CN, we have helped define the parameters which should guide investigations into the immunosuppressive and toxic effects of CsA. In many cases the experiments have already been done. With guidance from *in vivo* and clinical data, we can now revisit these questions with experiments that use relevant amounts of CsA.

One goal at the beginning of this project was to develop or identify a method by which clinicians could make rational decisions regarding immunosuppression in individual patients. While the CN data presented have provided significant insights into how CsA works in populations of patients, at present the CN assay shows too much internal variability to make individual measurements clinically meaningful. Work designed to reduce the variability associated with the clinical samples should lead to important clinical trials in which the value of CN monitoring can be properly addressed.

The central role of CN in lymphocyte stimulation makes CN and FK-506 excellent probes for dissecting the relative importance of the many intracellular signaling events described to date. Moreover the demonstration that CN inhibition is widespread during CsA therapy presents opportunities for studying gene activation and stimulus-dependent degranulation events in a variety of cell types. For example, the function of CN in endothelial cell activation during inflammation and injury, and the impact of CsA treatment on these activities, may be helpful in understanding some of the events mediated by EC such as leukocyte recruitment and adhesion, vasomotion and coagulation.

Chapter Seven

Conclusions

The results presented in this thesis demonstrate that:

1. CN activity could be measured in freshly isolated peripheral blood leukocytes and in the homogenates of murine organs
2. CsA inhibited CN activity in all circulating human blood cell types. The degree of inhibition, reflected by the IC_{50}, showed that all cell populations were equally sensitive to CsA, with the exception of RBC, for which 10 fold more CsA was required to inhibit CN.
3. CsA inhibited CN activity in homogenates of all murine organs tested except brain. *In vivo* inhibition of CN occurred at higher blood concentrations than those required to inhibit CN *in vitro*. Brain was not affected by *in vitro* or *in vivo* CsA.
4. CN inhibition was demonstrated in PBMC from CsA-treated patients, but there was less inhibition compared to the effect of similar concentrations of *in vitro* CsA.
5. The discrepancy between CsA inhibition of CN in PBMC in patients and the inhibition of CN in PBMC in culture medium was not due to recovery during clinical specimen handling, but was due to the distribution of CsA to non-PBMC sites in whole blood, leading to a 10 fold reduction in CsA accumulation in PBMC compared to PBMC in medium.
6. Following an oral CsA dose in patients, CN inhibition rose and fell concomitant with the rise and fall of CsA blood levels.

7. PBMC exposed to CsA were unlikely to recover CN activity *in vitro* in the absence of other non-PBMC binding sites.
8. CN recovery *in vitro* was temperature sensitive, protein synthesis independent and required the efflux of CsA from the cells, and was facilitated by the presence of RBC or αCsA Ab.
9. CsA efflux was facilitated in a cell line by the high expression of surface P glycoprotein.
10. CsA efflux in PBMC was not blocked by PGP inhibitors, indicating that there are at least two mechanisms of CsA efflux in mammalian cells.
11. In mice rejecting an allogeneic tumor, CsA inhibition of IFN-γ was quantitatively similar to the inhibition of CN activity.
12. In PBMC exposed to CsA *in vitro*, the inhibition of NFAT activation and DNA binding, IFN-γ and IL-2 mRNA induction, IFN-γ secretion and leukocyte proliferation in an MLR were all quantitatively linked to the degree of CN inhibition, indicating that CN activation is the rate limiting step in lymphocyte activation.

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